DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF SALBUTAMOL SULPHATE, GUAIFENESIN AND AMBROXOL HYDROCHLORIDE IN ORAL LIQUID DOSAGE FORM

Dissertation Submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
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In partial fulfillment for the award of the degree of

MASTER OF PHARMACY

(PHARMACEUTICAL ANALYSIS)

Submitted by

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Under the guidance of

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CERTIFICATE

This is to certify that the work embodied in this dissertation entitled "**DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF SALBUTAMOL SULPHATE, GUAIFENESIN AND AMBROXOL HYDROCHLORIDE IN ORAL LIQUID DOSAGE FORM",** submitted in partial fulfillment to the requirement for the award of degree of **MASTER OF PHARMACY** in the Department of Pharmaceutical Analysis, The Tamilnadu Dr. M.G.R. Medical University, Chennai, at Nandha College of Pharmacy, Erode, is a bonafied work carried out by **Reg. No.261530403** under my guidance and supervision during the academic year 2016-2017.

Dr.T.Prabha, M.Pharm., Ph.D., PDF.,

Place : Erode

Date :

EVALUATION CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF SALBUTAMOL SULPHATE, GUAIFENESIN AND AMBROXOL HYDROCHLORIDE IN ORAL LIQUID DOSAGE FORM",** submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, was carried out by **Reg. No. 261530403** as a Partial fulfillment for the award of degree of **MASTER OF PHARMACY** in the Department of Pharmaceutical Analysis under direct supervision of Dr. T. Prabha, M.Pharm., Ph.D., PDF., Head of the Department, Department of Pharmaceutical Analysis, Nandha College of Pharmacy, Erode-638052**,** during the academic year 2016-2017. The work is original and has not been submitted in part or full for any degree or diploma of this or other university.

INTERNAL EXAMINER **EXTERNAL EXAMINER**

DECLARATION

The research work embodied in this dissertation work entitled "**DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF SALBUTAMOL SULPHATE, GUAIFENESIN AND AMBROXOL HYDROCHLORIDE IN ORAL LIQUID DOSAGE FORM"** was carried out by me in the Department of Pharmaceutical Analysis, Nandha College of Pharmacy, Erode, under the direct supervision of **Dr**. **T. Prabha, M.Pharm., Ph.D., PDF.,** Professor, **Department of Pharmaceutical Analysis,** Nandha College of Pharmacy, Erode – 638052.

This dissertation submitted to **The Tamilnadu Dr. M.G.R Medical University, Chennai,** as a partial fulfillment for the award of **Degree of Master of Pharmacy** in Pharmaceutical Analysis during the academic year 2016 – 2017.

The work is original and has not been submitted in part or full for the award of any other Degree or Diploma of this or any other University.

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LIST OF ABBREVIATIONS USED

ICH	-	International Conference on Harmonization
IP	-	Indian Pharmacopoeia
USP	-	United States Pharmacopoeia
BP	-	British Pharmacopoeia
λ	-	Lambda
µg/mL	-	Microgram Per Millilitre
mg/mL	-	Milligram Per Millilitre
mL	-	Millilitre
mM	-	Milli Mole
nm	-	Nanometer
pH	-	Negative Logarithm of Hydrogen Ion
%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
RP-HPLC	-	Reverse Phase -High Performance Liquid Chromatography
RT	-	Retention Time
S.D	-	Standard Deviation
UV-VIS	-	Ultraviolet - Visible
IR	-	Infra Red
NMR	-	Nuclear Magnetic Resonance
AUC	-	Area Under Curve
˚C	-	Degree Celsius
µL	-	Microlitre
µ	-	Micron
v/v/v	-	Volume/Volume/Volume
SIAM	-	Stability indicating assay method
SAB	-	Salbutamol
GUA	-	Guaifenesin
AMB	-	Ambroxol
NaH_2PO_4	-	Sodium dihydrogen Orthophosphate
TEA	-	Triethyl Amine

CONTENTS

1. INTRODUCTION

Pharmaceutical Analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. The pharmaceutical analysis comprises the procedures necessary to determine the "identity, strength, quality and purity" of such compounds. It also includes the analysis of raw material and intermediates during manufacturing process of drugs.

1.1. TYPES [1]

Pharmaceutical analysis includes both qualitative and quantitative of drugs and pharmaceutical substances starts from bulk drugs to the finished products.

1.1.1. Qualitative analysis

- Qualitative inorganic analysis seeks to establish the presence of a given element or inorganic compound in a sample.
- Qualitative organic analysis seeks to establish the presence of a given functional group or organic compound in a samples

1.1.2 Quantitative analysis[2]

Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Importance of analytical chemistry is to gain information about the qualitative and quantitative composition of substance and chemical species, that is, to find out what a substance is composed of and exactly how much it is present

For the past two decades, the pharmaceutical analyst has been a spur in development of analytical techniques for various medicinal principles both in pure and combined state and also in finished products. Some of the methods shine in modern technological and electronics and microprocessor-based developments have really shown faster and best results with more accuracy than the conventional methods.

There are various methods used for quantitative analysis of mixtures. One of them is spectrophotometry, which utilizes the measurement of intensity of electromagnetic radiation emitted or absorbed by the analyte. Another technique which has gained large popularity during last decade is high performance liquid chromatography. The technique is very much useful to pharmaceutical analyst in analyzing complex formulation containing number of ingredients as it permits simultaneous separation and determination of components of mixture.

1.2. METHODS OF ANALYSIS

1.2.1. Generally analytical methods are classified as follows:

1. Chemical methods
2. Instrumental methods

a) Chemical methods

1. Volumetric method
2. Gravimetric method

b) Instrumental methods:

The analytical chemist, to save time, to avoid chemical separation or to obtain increased accuracy may use instrumental methods. This can be classified into:

1. Spectrophotometric methods – UV, IR, NMR etc.
2. Fluorimetric methods
3. Polarimetric method
4. Flame photometric method
5. Turbidimetric method
6. Chromatographic method
7. Refractrometric method
8. Thermal method
9. Electro chemical method

1.3. INTRODUCTION TO CHROMATOGRAPHY[3]

The word is obtained from the Greek word *chroma* + *graphia*, literally "colour writing". Chromatography is a technique for analyzing mixtures of gases, liquids or solutes by exploiting differences in their distribution between a stationary and a mobile phase. Chromatography is a fundamental technique in the detection, identification and quantization of chemical species. It comes in two basic formats, planar and column chromatography.

1.3.1. Classification of chromatography based on stationary phase

1.3.1.1. Planar Chromatography

The origins of planar chromatography can be traced back to the practice amongst dyers of testing pigments by placing a drop on paper and observing the colours as the drop spreads. The scientific use of paper chromatography can be traced to the mid-19th century. The important separation of amino acids and peptides by paper chromatography was developed in 1994 by Consden, Gordon, and Martin. Thin layer chromatography (TLC) was developed in 1938 by Izmailov and Shraiber based on Mikhail Tswett's earlier description of column chromatography.

1.3.1.2. Column Chromatography

Russian botanist Mikhail Tswett's invented column chromatography in 1906 as a means of studying plant pigments, but it soon became clear that the technique provided a means for separating many complex homogeneous mixtures into their individual components. Today instrumental chromatographic techniques are essential tools in areas such as chemistry, biology, medicine, forensic science, manufacturing and the environment.

Table 1 Classification of column chromatographic methods [2]

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
Liquid Chromatography (LC) (mobile phase: liquid)	Liquid-Liquid or partition	Liquid adsorbed on a solid	Partition between immiscible liquids
	Liquid-Bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Liquid-Solid or adsorption	Solid	Adsorption
	Ion exchange size exclusion	Ion-exchange resin Liquid in interstices of a polymeric solid	Ion exchange Partition/sieving
Gas Chromatography (GC) (mobile phase: gas)	Gas-liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-Solid	Solid	Adsorption
Supercritical-fluid chromatography (SFC) (mobile phase: Super critical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

1.3.2. Classification of chromatography based on mobile Phase

Chromatographic methods are also classified depending upon the medium of the mobile phase. There are two methods under this category:

1.3.2.1. Gas chromatography

Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium). The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical, environmental monitoring and remediation, and industrial chemical fields. It is also used extensively in chemistry research.

1.3.2.2. Liquid chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC).

1.3.2.3. Supercritical Fluid Chromatography (SFC)

SFC is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure. SFC is a form of normal phase chromatography that is used for the analysis and purification of low to moderate molecular weight, thermally labile molecules. It can also be used for the separation of chiral compounds. Principles are similar to those of high performance liquid chromatography (HPLC), however SFC typically utilizes carbon dioxide as the mobile phase; therefore the entire chromatographic flow path must be pressurized.

1.3.3. Classification of chromatography based on the mechanism of separation[4]

1.3.3.1. Adsorption chromatography

It involves the interaction between the sample molecule and the stationary phase. It is a competitive situation in which the molecules of the mobile phase and the solute are in competition for discrete adsorption sites on the surface of the column. Interaction between a solute molecule and the adsorbent surface is optimum when solute functional groups exactly overlap these adsorption sites. The adsorbent may be packed in a column e.g. silica, alumina.

The separation is achieved by changes in the composition of mobile phase. It is used for analysis of non-ionizing, water insoluble compounds.

1.3.3.2. Bonded phase chromatography

It is widely used column packing for liquid-liquid partition chromatography with chemically bonded, organic stationary phases. Partition occurs between the bonded phase and a mobile liquid phase. Bonded phase supports are made from silica by the covalent attachment of an organic hydro carbon moiety to the surface. A stationary phase chemically bonded to a support that is used for the separation. It is the most commonly used LC mode. The most popular support used is micro particulate silica gel. An organosilane, such as octadecyl (for reversed-phase chromatography) is the most accepted type of bonded phase.

1.3.3.3. Normal phase chromatography

Also known as Normal phase HPLC (NP-HPLC), in this method separation of analyte is based on polarity, it was the first kind of HPLC that chemists developed. NP-HPLC uses a polar stationary phase and a non-polar mobile phase and works effectively for relatively polar analyte. The polar analyte associates with and is retained by the polar stationary phase. Adsorption strength increase with increased analyte polarity and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time.

1.3.3.4. Size exclusion chromatography (SEC)

It is a chromatographic method in which particles are separated based on their size or in more technical terms their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. When an aqueous solution is used to transport the sample through the column the technique is known as gel filtration chromatography. The name gel permeation chromatography is used when an organic solvent is used as a mobile phase. The main application of gel filtration chromatography is the fractionation of proteins and other water-soluble polymers, while gel permeation chromatography is used to analyze the molecular weight distribution of organic-soluble polymers. Polymer chemists typically use either silica or cross linked polystyrene medium under a higher pressure. This media are known as the stationary phase.

1.3.3.5. Ion-exchange chromatography

It is a process that allows the separation of ions and polar molecules based on the charge properties of the molecules. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids. It is often used in protein purification, water analysis and quality control. Ion exchange chromatography retains analyte

molecules based on coulomb (ionic) interactions. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography. The ionic compound consisting of the cationic species M+ and the anionic species B- can be retained by the stationary phase.

1.3.3.6. Bio-affinity chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction and the hydrogen bond. An efficient bio specific bond is formed by a simultaneous and concerted action of several of these forces.

1.3.3.7. Hydrophobic Interaction Chromatography (HIC)

Hydrophobic Interaction Chromatography is a separation technique uses the properties of hydrophobic to separate proteins from one another. In this type of chromatography, hydrophobic groups such as phenyl, octyl or butyl are attached to the stationary column. Proteins that pass through the column that have hydrophobic amino acid side chains on their surfaces are able to interact with and bind to the hydrophobic groups on the column. HIC separations are often designed using the opposite conditions of those used in ion exchange chromatography. In this separation, a buffer with a high ionic strength, usually ammonium sulfate is initially applied to the column. The salt in the buffer reduces the solvation of sample solutes thus as solvation decreases, hydrophobic regions that become exposed are adsorbed by the medium. The stationary phase is designed to form hydrophobic interactions with other molecules.

Selection of suitable method for estimation of drug in dosage forms is an impending challenge for an analytical chemist. The method so selected should provide analytical data as accurate as required, technically sound, defensible with low level of uncertainty and above all amenable to routine laboratory use and capable of giving reproducible results. HPLC has become the back bone of the biotechnology and pharmaceutical industries where it is used to identify, characterize and purify molecules at all stages of a process, from R&D to quality assurance and validation.

1.3.4. Why HPLC having high importance compared with other techniques?

Different types of chromatographic techniques are available to analyze the samples, but one of the technique very familiar, is High Performance Liquid Chromatography. HPLC technique is not only useful for separation, but also useful for qualifying (identifying) and quantifying the small and neutral molecules also. A few microgram of sample (at the extreme, even less than a nanogram) is enough to ensure the required accuracy. Secondly, HPLC separations are usually relatively fast, precise, accurate and an analysis can be completed in short span possibly in a few seconds. Another advantage of these techniques is relative simplicity and ease of operation compared with other instrumental techniques. If the established procedure is well controlled and the apparatus is maintained under calibrated condition, good accuracy and precision can be achieved.

1.4. ANALYTICAL METHODS DEVELOPMENT

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods.

1.4.1 Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias,
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for the quantitation of the drug in biological fluids may not be available,

- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

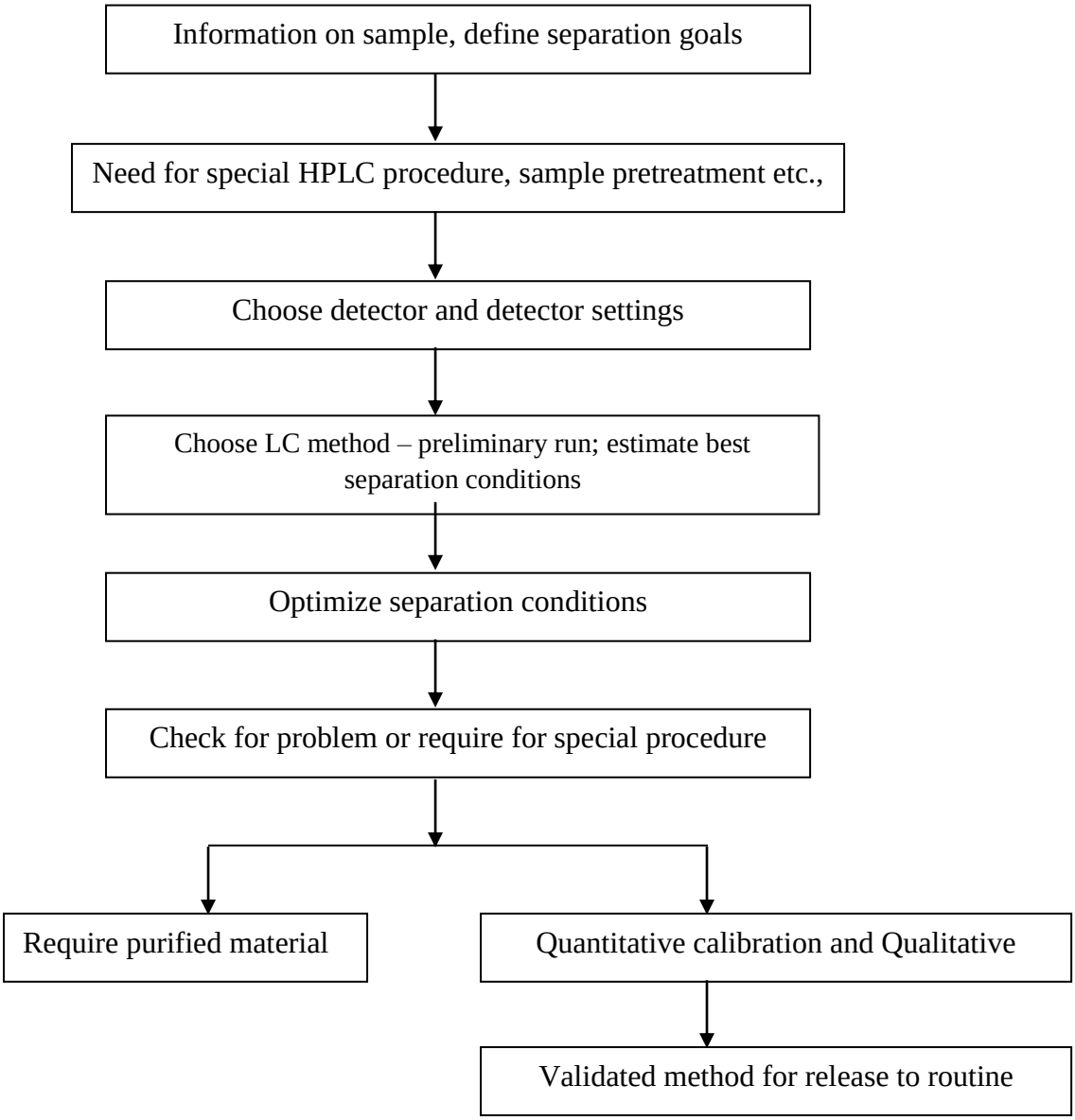
1.5. HPLC METHODS OF ANALYSIS FOR DRUGS IN COMBINATION

Most of the drugs in multi-component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less),
- Greater sensitivity (various detectors can be employed),
- Improved resolution (wide variety of stationary phases),
- Reusable columns (expensive columns but can be used for many analysis),
- Ideal for the substances of low volatility,
- Easy sample recovery, handling and maintenance,
- Instrumentation tends itself to automation and quantitation (less time and less labor),
- Precise and reproducible,
- Calculations are done by integrator itself,
- Suitable for preparative liquid chromatography on a much larger scale.

1.6. HPLC METHOD DEVELOPMENT [5]

HPLC method development seems complex. The process is influenced by the nature of the analytes and generally involves the following steps:



1.7. INSTRUMENTATION OF HPLC[2,3,4]

The general instrumentation for HPLC incorporates the following components,

- Solvent reservoir.
- Pump.
- Sampling valves or loops
- Guard column
- Pressure gauge
- Analytical column
- Detector
- Data acquisition system

Figure 1 Block diagram of a HPLC instrument

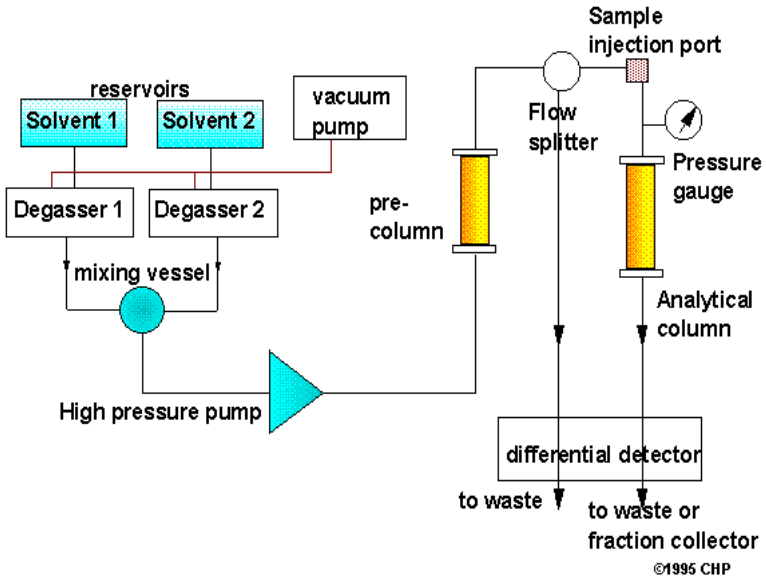
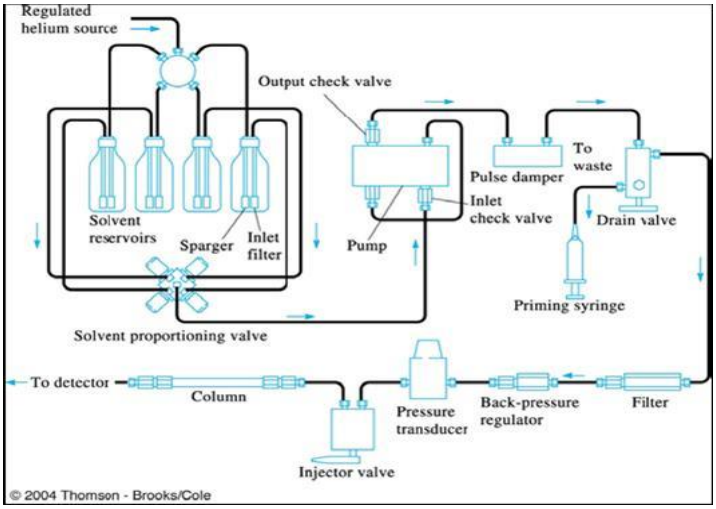


Figure 1 Schematic diagram of a HPLC instrument



1.8. TYPES OF HPLC BASED ON MODES OF SEPARATION

- ❖ NP-HPLC
- ❖ RP-HPLC

In the **normal phase mode**, the stationary phase is a polar substance such as polyethylene glycol or the untreated silica surface itself, and the mobile phase is non polar (e.g. hexane) under these circumstances polar compounds retarded preferentially and non polar substances elute more quickly.

In **reversed phase mode**, the stationary phase is non polar (e.g. ODS) and the mobile phase is polar, usually a mixture of water, methanol and/or acetonitrile. Non polar compounds are retained more strongly, while polar solutes elute first. Reversed phase separations are the most frequently used methods in HPLC.

Table 2 Comparison of NP – HPLC and RP – HPLC

Parameter	**Normal**	**Reversed**
Packing polarity	High	Low
Solvent polarity	Low	High
Elution order	Non-polar first, then polar	Polar first, then non-polar
Effect of increasing solvent polarity	Decreases retention time	Increases retention time

1.9. INTRODUCTION TO RP – HPLC[6]

Separation by RP – HPLC is similar to the extraction of different compounds from water into an organic solvent, where more hydrophobic (non - polar) compounds extract into the non - polar phase. The column (typically C8 and C18 bonded phase) is less polar than the water - organic phase. Sample molecules partition between the polar mobile phase and non – polar C8 and C18 stationary phase and more hydrophobic (non - polar) compounds are retained more strongly. Polar compounds are less strongly held and elute from the column first and vice versa. In RP – HPLC the retention of a compound is determined by its polarity, experimental conditions, mobile phase, column and temperature. RP – HPLC columns are efficient, stable and reproducible.

1.9.1. Mobile phase effects

Retention is preferable adjusted by changing mobile phase composition or solvent strength. In this the retention is less for stronger, less polar mobile phase. Solvent strength depends on the choice of organic solvent and its concentration in the mobile phase.

1.9.2. Mobile phase strength

1. In RP-HPLC solvent strength varies as
2. Water < Methanol < Acetonitrile < Ethanol < Tetrahydrofuran < Propanol < Methylene chloride.
3. Acetonitrile is best initial choice of solvent and Acetonitrile and Water mixture used for UV detection.

1.10. VARIOUS METHODS OF QUANTITATIVE ANALYSIS IN HPLC

The sample or solute is analyzed quantitatively in HPLC by either peak height or peak area measurements. Peak areas are proportional to the amount of constant rate. Peak heights are proportional to the amount of material only when peak width are constant and are strongly affected by the sample injection techniques. Once the peak height or the peak areas are measured, there are five principle evaluation methods for quantifying the solute[3,7].

1.10.1. Calibration by Standards

Calibration curves for each component are prepared from pure standards, using identical injection volumes of operating conditions for standards and samples. The concentration of solute is read from its curve if the curve is linear.

$$X = K \times Area.$$

Where, X = Concentration of solute.

K = Proportionality constant (slope of the curve).

In this evaluation method only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of a given detector differs for each molecular type of compounds.

1.10.2. Internal Standard Method

In this technique a known quantity of the internal standard is chromatographed and area versus concentration is ascertained. Then a quantity of the internal standard is added to the raw sample prior to any sample pretreatment or separation operations.

The peak area of the standard in the sample run is compared with the peak area when the standard is run separately. This ratio serves as a correction factor for variation in sample size for losses in any preliminary pretreatment operations or for incomplete elution of the sample. The material selected for the internal standard must be completely resolved from adjacent sample components, must not interfere with the sample components and must never be present in samples.

$$\text{Area ratio} = \frac{\text{Area of sample}}{\text{Area of internal standard}}$$

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of internal standard}} \times \text{Concentration of standard}$$

1.10.3. Area Normalization

The technique is often used for the sample having identical components. It is used to evaluate the absolute purity of the sample. The procedure is to total up the areas under all peaks and then calculate the percentage of the total area that is contributed by the compound of interest. For this method the entire sample must be eluted, all components must be separated and each peak must be completely resolved.

1.10.4. Standard Addition Method

If only few samples are to be chromatographed, it is possible to employ the method of standard addition(s). The chromatogram of the unknown analyte is recorded, then a known amount of analyte(s) is added and the chromatogram is repeated using same reagents, instruments and same conditions. From the increase in the peak area (or) peak height, the original concentration can be computed by interpolation.

The detector response must be a linear function of analyte concentration and yield no signal at zero concentration of the analyte. Sufficient time must elapse between addition of the standard and actual analysis to allow equilibrium of added standard with any matrix interferon.

If an instrumental reading (area/height) 'R_x' is obtained, from a sample of unknown 'x' and a reading 'R_t' is obtained from the sample to which a known concentration 'a' of analyte has been added, then 'x' can be calculated from.

$$\frac{x}{x+a} = \frac{R_x}{R_t}$$

A correction for dilution must be made if the amount of standard added changes the total sample volume significantly. It is always advisable to check the result by adding at least one other standard.

1.10.5. External Standard Method

It employs a separate injection of a fixed volume of sample and standard solution. The peaks are integrated and concentration is calculated.

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard}$$

The selection of suitable chromatographic (HPLC) system for a given mixtures of solutes cannot be made with certainty and must be confirmed by experiment. If the chemical nature of the sample components is known, then the phase system can be selected from the literature references. If nothing is known about the chemical nature of sample, then the sample solubility will give some indication as to which chromatographic method to employ. The essential parts of high performance liquid chromatographic system are solvent reservoir, pump, injection port, column, detector and recorders.

1.11. METHOD VALIDATION

Method validation can be defined as establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

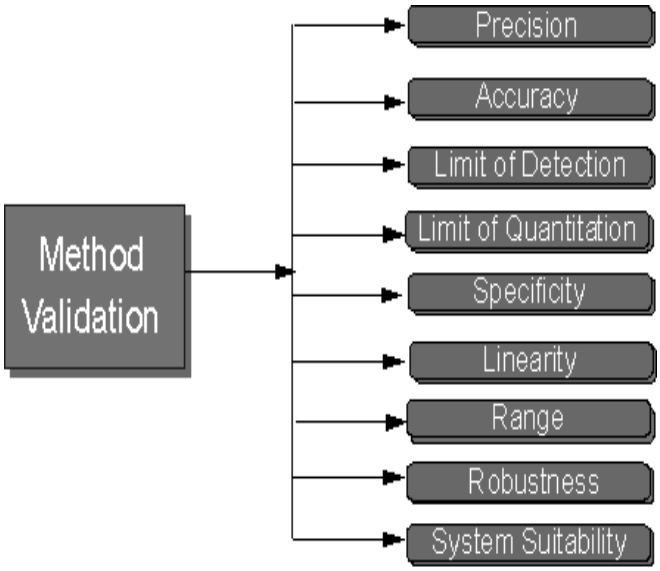
Method Validation, however, is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

1.11.1. Purpose of Validation

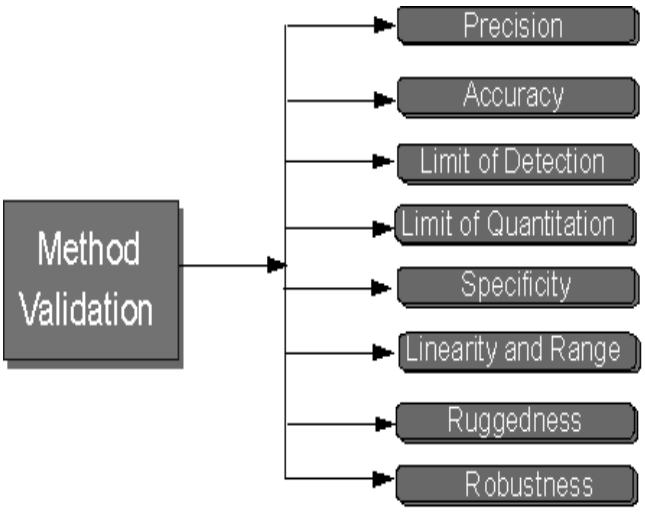
- Enable the scientists to communicate scientifically and effectively on technical matter.
- Setting the standards of evaluation procedures for checking compliance and taking remedial action.
- Economic: Reduction in cost associated with process sampling and testing.
- As quality of the product cannot always be assured by routine quality control because of testing of statistically insignificant number of samples.
- Retrospective validation is useful for trend comparison of results compliance to CGMP/CGLP.
- Closure interaction with Pharmacopoeial forum to address analytical problems.
- International Pharmacopoeial harmonization particularly in respect of impurities determination and their limits.

1.11.2. Guidelines for method validation [7,8,9]

ICH Method Validation



USP Method Validation



Typical validation characteristics which should be considered and their definitions are given below.

1.11.2.1. Accuracy

The closeness of agreement between the value, which are accepted either as a conventional true value or an accepted reference value and the value found. Accuracy is represented and determined by recovery studies.

1.11.2.2. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous

sample under the prescribed conditions. A more comprehensive definition proposed by the ICH divides precision into three types

1. Repeatability
2. Intermediate Precision
3. Reproducibility

1. Repeatability

It is the precision of a method under the same operating conditions over a short period of time. One aspect of this is instrumental precision. A second aspect is sometimes termed intra-assay precision and involves multiple measurements of the same sample by the same analyst under the same conditions.

2. Intermediate precision

It is the agreement of complete measurements when the same method is applied many times within the same laboratory. This can include full analysis on different days, instruments or analysts, but would involve multiple preparations of samples and standards.

3. Reproducibility

It examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments. Precision often is expressed by the standard deviation or relative standard deviation of the data set.

1.11.2.3. Range

The range of a method can be defined as the upper and lower concentrations for which the analytical method has adequate accuracy, precision and linearity. The range of concentrations examined will depend on the type of method and its use.

1.11.2.4. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Assuring specificity is the first step in developing and validating a good method. If specificity is not assured, method accuracy, precision and linearity all are seriously compromised. Method specificity should be reassessed continually during validation and subsequent use of the method.

1.11.2.5. Linearity

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity can be assessed by performing single measurements at several analyte concentrations. A linearity correlation coefficient above 0.999 is acceptable for most methods, especially for major components in

assay methods. The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample.

1.11.2.6. Detection Limit

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

a) Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

b) Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

c) Based on the Standard Deviation of the Response and the Slope

The detection limit (LOD) may be expressed as

$$LOD = 3.3\,\sigma / S$$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

d) Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

e) Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

1.11.2.7. Quantitation Limit

Several approaches for determining the quantitation limit are possible; depending on either the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

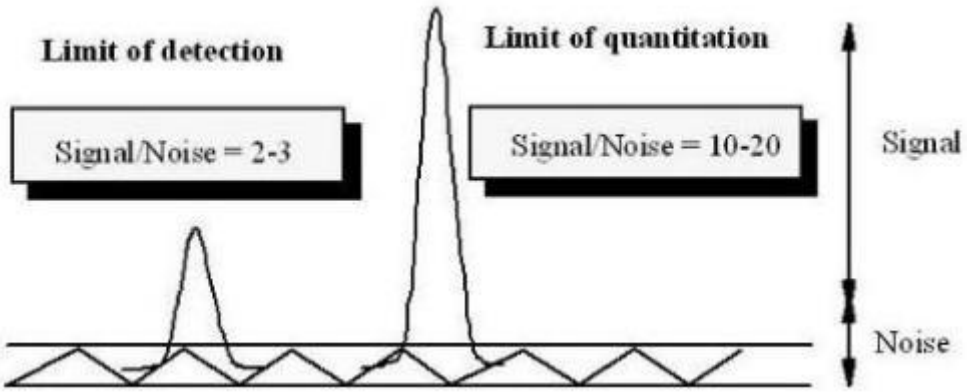
a) Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

b) Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.



c) Based on the Standard Deviation of the Response and the Slope

Quantitation Limit (LOQ) may be expressed as

$$LOQ = 10\,\sigma/S$$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

d) Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

e) Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

1.11.2.8. Ruggedness

Method ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents and so on. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

1.11.2.9. Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". The most important aspect of robustness is to develop methods that allow for expected variations in the separation parameters.

Table 3

Acceptance criteria of validation for HPLC

S.No	Characteristics	Acceptance Criteria
1	Accuracy	Recovery 98-102% with 80, 100, 120% spiked sample.
2	Precision	
2a	Repeatability	RSD < 2%
2b	Intermediate precision	RSD < 2%
3	Specificity/ selectivity	No interference
4	Detection Limit	S/N > 2 or 3
5	Quantitation Limit	S/N > 10
6	Linearity	r > 0.999
7	Range	80-120%
8	Stability	>24h or > 12h

1.12. SYSTEM SUITABILITY PARAMETERS[9]

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

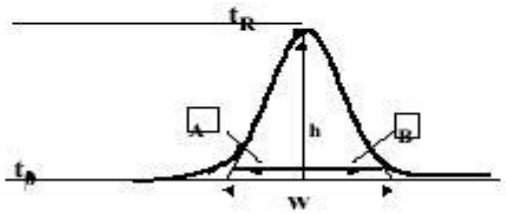
The parameters that are affected by the changes in chromatographic conditions are,

- Retention or Capacity factor (K_A)
- Resolution (R_s)
- Selectivity (α)
- Column efficiency (N) and
- Peak asymmetry factor (A_s)

I.RETENTION OR CAPACITY FACTOR (K_A)

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} \qquad k' = \phi \frac{C_s}{C_m}$$



The retention of a drug with a given packing material and eluent can be expressed as retention time or retention volume, but both of these are dependent on flow rate, column length and column diameter. The retention is best described as a column capacity ratio (K), which is independent of these factors. The column capacity ratio of a compound (A) is defined as

$$K_A = \frac{V_A - V_0}{V_0} = \frac{t_A - t_0}{t_0}$$

Where,

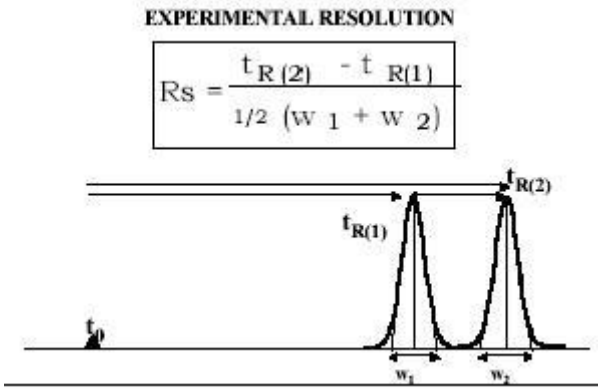
V_A = Elution volume of A

V_0 = Elution volume of a non retained compound (void volume)

At constant flow rate, retention times (t_A and t_o) can be used instead of retention volumes. Retention data is sometimes expressed, relative to a known internal standard (B). The ratio of retention times (t_A/t_B) can be used, but the ratio of adjusted retention times $\left(\frac{t_A - t_o}{t_B - t_o}\right)$ is better when data need to be transferred between different chromatographs.

The values of k' of individual bands increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water/organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k' of the bands by a factor of 2-3.

II.Resolution (R_s)



The resolution, R_s of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 2.0. It is calculated by using the formula,

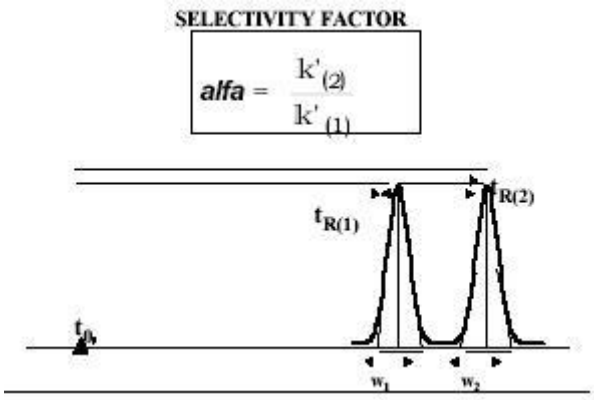
$$R_f = \frac{Rt_2 - Rt_1}{0.5\,(W_1 + W_2)}$$

Where,

R_{t1} and R_{t2} are the retention times of components 1 and 2.

W_1 and W_2 are peak widths of components 1 and 2.

III.Selectivity (α)



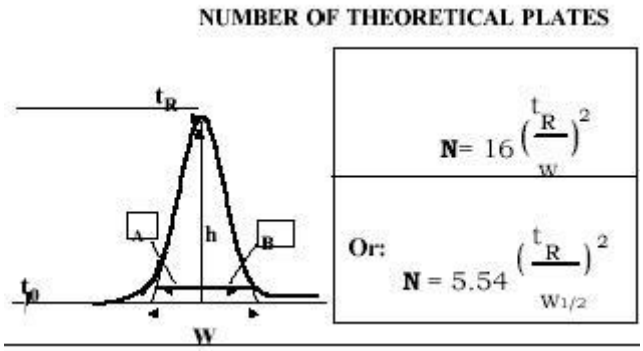
The selectivity (or separation factor) α, is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peaks respectively.

IV.Column efficiency

Efficiency N of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance.

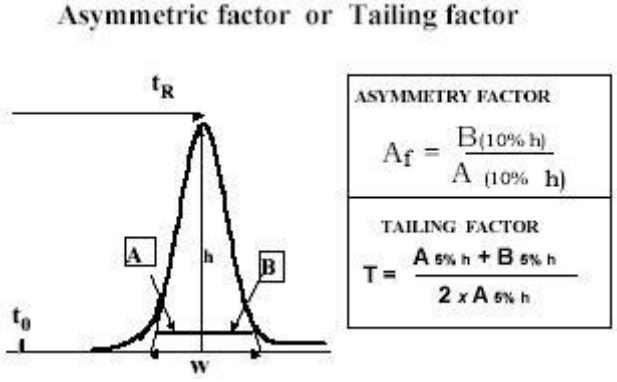


Columns with N ranging from 5,000 to 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16 \frac{Rt^2}{W^2}$$

Where, R_t is the retention time and W is the peak width.

V.Peak asymmetry factor (A_s)



Peak asymmetry factor A_s can be used as a criterion of column performance. The peak half width b of a peak at 10 % of the peak height divided by the corresponding front half width a gives the asymmetry factor.

$$A_s = \frac{b}{a}$$

1.13. STATISTICAL PARAMETERS[10]

i) Regression equation

The linear relationship is characterized by at tendency of the points of the scattered diagram to cluster along a straight line known as the regression line.

$$Y=a + bX$$

It is used to describe the dependence of one characteristic (Y) up on the other characteristic (X), both X,Y represent values of two characters a,b are two constants it will be evident that two regression lines can be computed for every set of data-one each to describe the dependence of one character to another. b is known as regressive coefficients which shows change expected in Y for unit change in X, it is dependence of Y & X; b is the regressive coefficient of Y& X. The regressive coefficient of b is estimated,

$$b = \frac{\Sigma\left(x - \bar{x}\right)\left(y - \bar{y}\right)}{\Sigma\left(x - \bar{x}\right)^2}$$

b = the slope of the regression line and is calculated by this formula

x = an arbitrarily chosen value of the predictor variable for which the corresponding value of the criterion variable is desired.

ii) Correlation coefficient

A measure of the strength of the relationship between two variables is provided by the coefficient of correlation denoted by r, if the relationship between the two variables is of the linear form. It is also called the coefficient of linear correlation.

iii) Pearson's correlation

The correlation coefficient calculation for data values should be +1 or -1 where the values of

Correlation coefficient is +1 – positive

Correlation coefficient is -1 – negative.

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{(\sum X^2 - \frac{(\sum X)^2}{N})(\sum Y^2 - \frac{(\sum Y)^2}{N})}}$$

Where, X – value of one character

Y – Value of another character

iv) Standard Deviation

It is the square root of the average of the squared deviations of the observations. From the arithmetic mean, it is used for measures of dispersion. It is denoted by

$$\text{Standard Déviation} = \sqrt{\frac{\Sigma(x-x)^2}{n-1}}$$

$$\text{R.S.D (\%)} = \frac{\text{S.D}}{\bar{x}} \times 100$$

Where Σ = Sum of observations

x ≡ Mean or arithmetic average ($\Sigma x / n$)

x = Individual observed value

$x - \bar{x}$ = Deviation of a value from the mean

n = Number of observations

v) Standard error of mean (S.E)

The population of standard deviation is not given, but the size of s is large. So the sample standard deviation is representing the population of standard deviation.

$$\text{S.E.} = \frac{\text{S.D}}{\sqrt{n}}$$

Where, S.D = Standard Deviation

n = No. of observations

1.14. STABILITY INDICATING METHOD[11]

A stability indicating method is a validated qualitative analytical procedure that can detect the cha nges with time in the properties of drug substance and drug product under defined storage conditions. A stability indicating assay method accurately measures the active ingredient without interference from other peaks and is sensitive enough to detect and qualify the degradation products/impurities. To develop a stability indicating method, stress testing in the form of forced degradation should be carried out at an early stage so that impurities and degradation products can be identified and characterized.

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of Stability-Indicating Assay Method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products.

Elaborate **definitions of stability-indicating methodology** are, however, provided in the United States-Food and Drug Administration (US-FDA) stability guideline of 1987 [5] and the draft guideline of 1998 [6].

Stability-indicating methods **according to 1987 guideline were defined** as the 'quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.'

This **definition in the draft guideline of 1998** reads as: 'validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference.'

According to FDA guidance document, a stability-indicating method is "a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability indicating method accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities."

HPLC has been very widely employed in stability studies due to its high-resolution capacity, sensitivity and specificity. Non-volatile, thermally unstable or polar/ionic compounds can also be analyzed by this technique. Therefore, most of the SIAMs have been established using HPLC.

1.14.1. Steps in the Development of Validated SIAMS

The practical steps involved in the development of SIAMs are discussed below:

Step I : Critical study of the drug structure to assess the likely decomposition route(s)

Step II : Collection of information on physicochemical properties

Step III : Stress (forced decomposition) studies

Step IV : Preliminary separation studies on stressed samples

Step V : Final method development and optimization

Step VI: Identification and characterization of degradation products, and preparation of standards

Step VII: Validation of SIAMs

ICH guideline Q1AR and the ICH's Common Technical Document [181] suggest the drug substance only subjected to stress conditions for the development of a SIAM.

1.14.2. Forced Degradation Studies

Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicating methods. These studies also provide information about the degradation pathways and degradation products that could form during storage. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development, manufacturing, and packaging, in which knowledge of chemical behavior can be used to improve a drug product.

Forced degradation studies (or stress testing) are discussed and differentiated from accelerated testing, which is done during formal stability testing, in **ICH Q1A (R2) [1]. The guidance states,**

"Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability-indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved."

As stated in ICH Q1A, forced degradation studies can be used as a predictive tool [3]. The initial purpose of these studies is to investigate stability-related properties of an API and understand the degradation products and pathways of the compound. They are also used to provide samples for the development of stability-indicating analytical methods for the API. The information gleaned from a forced degradation investigation can also be utilized in several other areas of development, including analytical development (methods development), formulation development (formulation choice and storage conditions), manufacturing/processing parameters (synthesis/salt selection of API and manufacture of formulations), safety/toxicological concerns (possible genotoxic degradation products), metabolism (identification of possible metabolites) and discovery (design of better or more stable APIs).

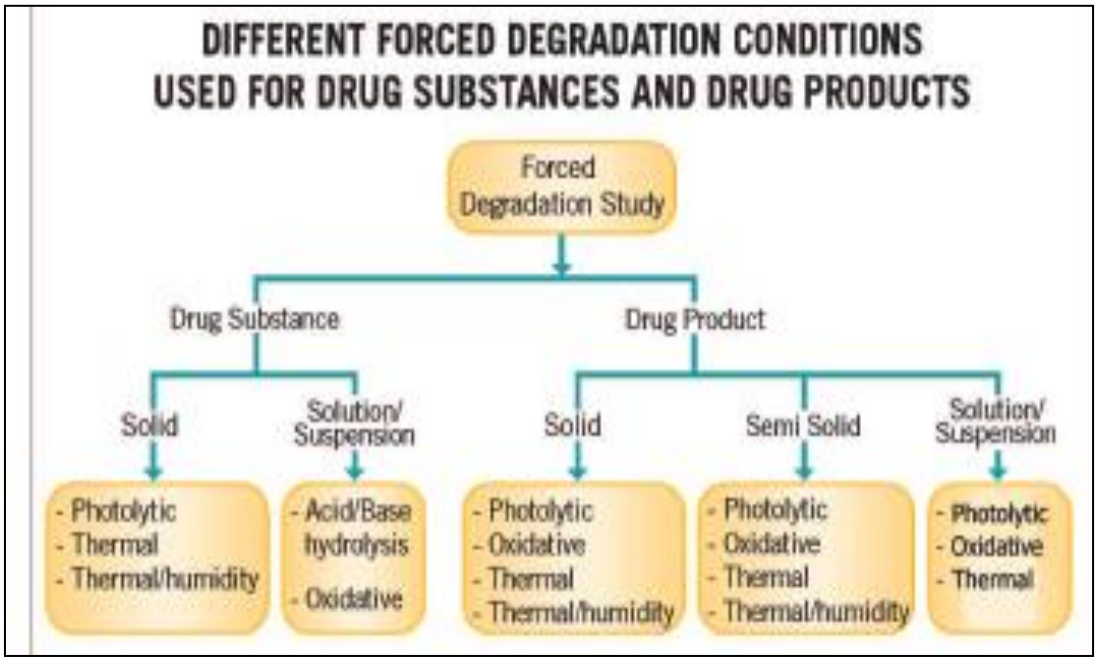
1.14.3 Forced degradation studies are carried out for the following reasons:

1. Development and validation of stability-indicating methodology
2. Determination of degradation pathways of drug substances and drug products
3. Discernment of degradation products in formulations that are related to drug substances versus those that are related to non–drug substances (e.g., excipients)
4. Structure elucidation of degradation products
5. Determination of the intrinsic stability of a drug substance molecule.

1.14.4. Degradation Studies of a Drug Substance

For degradation studies of a drug substance, FDA requests the following at the time of registration:

- Stressing the drug substance in solution or suspension at acidic and alkaline pH and under oxidation conditions
- Stressing the solid bulk drug substance at temperature and temperature - humidity conditions in excess of accelerated conditions
- Stressing the drug substance photolytically in the solid state and/or in solution



There are a number of common stresses which are used to

- pH (acid/base)

Chemical processes are often catalyzed by the presence of acids and bases. The exposure of materials to these can therefore accelerate degradation reactions.

- Temperature

In accordance to Arrhenius kinetics, increasing temperature increases the rate of any degradation process. Temperature is often used in conjunction with other stresses to increase reaction rates.

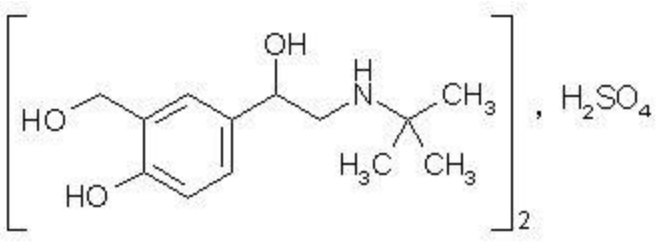
- Oxidation
- Concentration
- Light

For the stress conditions to provide the best compromise between overstressing and understressing the sample, the desired target for the extent of observed degradation is approximately 5-20%. Overstressing the sample may lead to further degradation of the primary degradants or it may generate irrelevant degradants that would never be seen in formal stability studies. At the other extreme, understressing may fail to generate important degradation products that may be generated in formal stability studies. The desired extent of degradation can be achieved by varying one or more of the stress conditions, e.g., exposure time, temperature, or concentration of stressing agent (acid, base, oxidizer, etc.).[12].

2. DRUG PROFILE

2.1 SALBUTAMOL SULFATE[21]

Molecular structure **:**



Chemical Name **:** (RS)- 4-[2-(tert- Butylamino)-1-hydroxyethyl)-2 (hydroxymethyl) Phenol

Molecular Formula **:** $(C_{13}H_{21}NO_3)_2.H_2SO_4$

Molecular weight **:** 576.7 g/mol

Therapeutic class **:** Selective beta2-adrenergic receptor agonist

Category **:** Bronchodilators, Sympathomimetic, Chronic obstructive, Pulmonary disease

Metabolism **:** Liver

Onset of action **:** <15 min (inhaled), <30 min (pill)

Duration of action **:** 2-6 hrs

Excretion **:** Kidney

Description **:** A white or almost white, crystalline powder

Solubility **:** Soluble in ethanol, sparingly soluble in water, very soluble in chloroform

Melting point **:** 147^0-149^0C

Half life **:** 3.8-6 hours

Loss on drying **:** Not more than 0.5%

Assay **:** 99.99% w/w

Storage **:** Store protected from light

Mechanism of action[13] **:** Salbutamol is a beta(2)-adrenergic agonist and thus it stimulateous Beta(2)-adrenergic receptors. Binding of albuterol to beta(2) receptors in the lungs results in relaxation of bronchial smooth muscles. It is believed that salbutamol increases cAMP. Production by activating adenylate cyclase, and the actions of salbutamol are mediated by cAMP. Increased intercellular cyclic AMP increases the activity of cAMP-dependent protein kinase A, which inhibits the phosphorylation of myosin and lowers intracellular calcium concentration .A lowered intercellular calcium concentration leads to a smooth muscle relaxation and bronchodilation.In addition to bronchodilation, salbutamol inhibits the release of broncho-constricting agents from mast cells, inhibits microvascular leakage, and enhances mucocilliary clearance.

Adverse effect[14] **:** Palpitation, Tachycardia, Chest discomfort, Headache, Tremor, Anxiety

Contraindication **:** Contraindicated in person with a history of,

1). Hypersentivity reaction

2). Urticaria

3). Angioedma

4). Tachyarrthymias

Marketed Formulation **:** 1). Tablet

2). Syrup

3). Inhaler

4). Nebulizer and

5). Intramuscular or Intravenous Injectable form

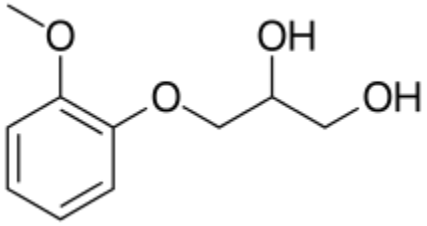
Brand name **:** 1). Accuneb

2). Proventil

3). Ventolin

2.2 GUAIFENESIN[21]

Molecular structure :



Chemical name : 3-(2-methoxyphenoxy) propane-1,2-diol

Molecular formula : $C_{10}H_{14}O_4$

Molecular weight : 198.2g/mol

Therapeutic category : 1). Expectorant

2). Muscle relaxing action

Description : White or almost white, crystalline powder

Solubility : Free soluble in ethanol, soluble chloroform, glycerol, water

Melting point : 78.5- 79°C

Half life : 1 hour

Absorption : Rapidly absorbed from GI tract

Metabolism : Rapidly hydrolysed (60% within 7 hours)

Excretion : Excreted in Urine

Loss on drying : Not more than 0.5%

Storage : Stored in tight containers at room temperature between 15˚C and 30˚C

Mechanism of action[15] : Guaifenesin may act as an irritant to gastric vagal receptor, And recruit efferent parasympathetic reflexes that causes Glandular exocytosis of a less viscous mucus mixture . Cough may be provoked. This combination may flush tenacious ,congealed mucopurulent material from obstructed small airways and lead to temporary improvement dyspnea or work of breathing.

Adverse effect[16] **:** 1). Dizziness

2). Headache

3). Nausea

4). Vomiting

5). Skin rash

6). Stomach pain

7). Urticaria (hives).

Contraindication **:** Contraindicated in patients hypersentivity to the drug

Marketed formulation **:** 1). Capsules

2). Tablets

3). Syrup

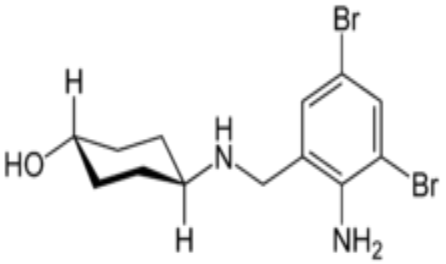
Brand name[17] **:**1). Mucinex

2). Fenesin IR

3). Robitussin

2.3 AMBROXOL HYDROCHLORIDE[21]

Molecular structure **:**



. HCL

Chemical name **:** Trans-4-[(2-amino-3,5-dibromobenzyl)amino]cyclohexanol Hydrochloride

Molecular formula **:** $C_{13}H_{19}Br_2ClN_2O$

Molecular weight **:** 414.566 g/mol

Therapeutical category[18] **:** Mucolytic agent, Expectorant

Description : A white or yellowish crystalline powder

Solubility : Sparingly soluble in water , soluble in methanol

Practically Insoluble in methylene chloride

Melting point : 233-234.5°C

Half life : 7-12 hours

Loss on drying : Not more than 0.5%

Protein binding : Approximately 90%

Storage : Store protected from light, temperature not exceeding 30°C

Mechanism of action[19] : Ambroxol is a mucolytic agent. Excessive nitric oxide (NO) is Associated with inflammatory and some other disturbances of Airway functions. NO enhances the activation of soluble Guanylate cyclase and cGMP accumulation. Ambroxol has been shown to inhibit the NO-dependent activation of soluble Guanylate cyclase.It is also possible that the inhibition of NO-dependent activation of soluble guanylate cyclase can suppress the excessive mucus secretion, therefore it lowers the phlegm viscosity and improves the mucocilliary transport of bronchial secretions.

Adverse effect[20] : 1). Occasional gastrointestinal side effect

2). Allergic reaction

3). Heart Burn

4). Dry mouth

5). Dyspepsia

6). Nausea

7). Vomiting

Very rarely,

i). Stevens-Johnson syndrome

Contraindication **:** Hypersentivity to the drug, Hereditary disorders of Carbohydrates tolerance (product contain lactose) Duodenal ulcer, convulsion. There is no absolute Contraindication but in patient with gastric ulceration relative caution should be observed.

Marketed formulation **:** 1). Tablet

2). Syrup

Brand name **:** 1). Acticol

2). Acolyt

3). Acocontin

4). Ambrodil

3. LITERATURE REVIEW

Literature review was carried out to enumerate the reported analytical methods for the selected drugs individually or in combination with other drugs.

Neelima *et al.*,[22] have developed the different analytical methods available for detection of Guaifenesin alone and in combination from various pharmaceutical formulations. They are many analytical techniques have been reported for simultaneous estimation of Guaifenesin and its combined pharmaceutical dosage form but only fewer methods have been reported for estimation of Guaifenesin alone. Chromatographic separation was performed on symmetry (C18) inertsil ODS 3V (4.6mm×150mm,4.5µm) column, with mobile phase comparising of mixture of phosphate buffer (pH 6.0,adjusted with NaOH) and acetonitrile in the ratio of 40:60%v/v, at the flow rate 1ml/min at detection wavelength of 220nm. Some of those techniques are UV spectrophotometry, high performance liquid chromatography (HPLC), high-performance thin layer chromatography (HPTLC), liquid chromatography - mass spectrometry (LC-MS), gas chromatography (GC),and ultraperformance liquid chromatography (UPLC). Amongst, various analytical methods are available for the quantification of single and multicomponent dosage forms.

Bhauvaneswara *et al.*,[23] have developed the present study a validated stability indicating RP HPLC method for quantification of GFS, LCZ and ABX was established. Compared with the reported methods, this method represents the first report about a stability indicating method for the determination of GFS, LCZ and ABX. With the proposed method a satisfactory separation of three drugs from the degradation products and impurities, extended linear range and rapid analysis time were carried out. C18 column (250×4.6 mm, 5 µm Particle Size) with the mobile phase consisting of 0.01 M Phosphate Buffer pH-4.5 adjusted with 1% of Ortho Phosphoric Acid: Acetonitrile (45:55% v/v). Detection was carried out at 254 nm. A high recovery of GFS, LCZ and ABX in formulation was achieved. The proposed method ensured a precise and accurate determination of GFS, LCZ and ABX in formulation. No interference from the excipients was noticed.

Ankit B. Chaudhary *et al.*,[24] have developed a Reverse Phase High Performance Liquid Chromatographic method was developed for the simultaneous estimation of Bromhexine Hydrochloride, Guaiphenesin and Chlorpheniramine Maleate. The separation was achieved by C18 column (250×4.6 mm, 5 µm Particle Size) with the mobile phase consisting of 0.01 M Potassium dihydrogen Phosphate Buffer pH-3 adjusted with 1% of Ortho Phosphoric Acid: Acetonitrile (40:60% v/v). Detection was carried out at 254 nm. RP-HPLC method gives retention time of Bromhexine Hydrochloride (BH), Guaiphenesin (GP) and Chlorpheniramine Maleate (CPM) was found to be 5.54min, 2.03min and 6.61 min respectively. The method has been validated for ICH Guideline. Linearity for Bromhexine Hydrochloride, Guaiphenesin and Chlorpheniramine Maleate were found in the range of 10-60 µg/ml, 125-750µg/ml and 5-30 µg/ml. The percentage recoveries obtained for BH, GP and CPM were found to be in range of 99.40-101.95%, 100.63-101.51% and 100.64-101.51% respectively in tablet. Limit of detection and Limit of quantification was found to be 1.449µg/ml and 4.830µg/ml for BH, 15.02µg/ml and 50.08µg/ml for GP and 0.795µg/ml and 2.651µg/ml for CPM respectively.

Hina Bagada *et al.*,[25] have developed the study is to develop simple, precise, accurate, rapid and sensitive Method of Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and High Performance Thin Layer Chromatography (HPTLC) for simultaneous estimation of Ambroxol HCl (AMB), Dextromethorphan HBr (DEX) and Guaifenesin (GUA) in Pharmaceutical Cough Cold Preparation and statistical comparison of both method was done. For RP-HPLC method Hibar RP-C18 (250mm × 4.6mm i.d. with particle size of 5µm) analytical column was used. The mobile phase composition used for RP-HPLC method was Acetonitrile - Methanol - 10mM Phosphate Buffer – 0.3% Triethyl Amine (25:15:60 v/v) and pH was adjusted to 3 using Orthophosphoric Acid. Flowrate was kept 1 mL/min. Detection was done at 205 nm using PDA detector. Linearity range for RP-HPLC method was found to be 3-10.5 µg/ml, 2-7 µg/ml and 10-35 µg/ml for AMB, DEX and GUA respectively. In HPTLC method Merck HPTLC plates precoated with 60F254 silica gel on aluminium plates were used as stationary phase. Mobile phase used in HPTLC method was Toluene – Methanol – Chloroform – Glacial Acetic Acid (6.5:1.5:1.5:0.5 v/v/v/v). Detection was done using Camag TLC scanner at 275 nm. Linearity range was found to be 1.5-3 µg/band, 1-2 µg/band and 5-10 µg/band for AMB, DEX and GUA respectively for HPTLC method.Statistical comparison of

RP-HPLC and HPTLC was done by application of t-Test to recovery data of both methods. Finally concluded that A novel RP-HPLC and HPTLC methods were developed for simultaneous estimation of AMB, DEX and GUA in syrup dosage form. Both the method gives the good resolution for all three drugs. The developed method was validated and found to be sensitive, accurate, specific and reliable for simultaneous estimation of AMB,DEX and GUA in syrup dosage form. The proposed method can be used effectively in routine analysis.

T. Manjula *et al.*,[26] have developed for simultaneous estimation of Levofloxacin hemihydrates (LEVH) and Ambroxol HCL (AMXL HCL) in pharmaceutical dosage form. Chromatographic separation was performed on symmetry (C18) inertsil ODS 3V (4.6mm×150mm,4.5µm) column,with mobile phase comparising of mixture of phosphate buffer (pH 6.0,adjusted with NaOH) and acetonitrile in the ratio of 35:65%v/v, at the flow rate 1ml/min at detection wavelength of 231nm.The retention times of LEVH and AMXL HCL were found to be and 2.464 mins respectively with a run time of 6 mins, with a resolution of 6.61.As per ICH guidelines the method was validated for linearity,accuracy,precision,limit of detection and limit of quantification,and ruggedness,robustness.Linearity of LEHV was found in the range of 20-60µg/ml and that for AMXL HCL was found to be 37.5-112.5µg/ml.The correlation coefficient for LEVH and AMXL HCL were 0.997 and 0.995 respectively.The LOD values for LEVH and AMXL HCL were 2.66µg/ml,0.88µg/ml and respectively .The LOQ values for LEVH and AMXL HCL were 8.06 and 0.29µg/ml respectively.This demonstrates that the developed method is simple,precise,rapid,selective,accurate and reproducible for simultaneous estimation of LEVH and AMXL HCL combined dosage form.

A. Porel *et al.*,[27] have developed the present study was the development and subsequent validation of a simple, precise and stability-indicating reversed phase HPLC method for the simultaneous determination of guaifensin, terbutaline sulphate and bromhexine hydrochloride in the presence of their potential impurities in a single run. The photolytic as well as hydrolytic impurities were detected as 3,5-dihydroxybenzoic acid, 3,5-dihydroxybenzaldehyde, 1-(3,5 dihydroxyphenyl)-2-[(1,1-dimethylethyl) amino]-ethanone from terbutaline, 2-methoxyphenol and an unknown impurity identified as(2RS)-3-(2-hydroxyphenoxy)-propane-1,2-diol from guaifenesin. The chromatographic separation of all the threeactive components and their impurities was achieved on Wakosil II column, using phosphate buffer (pH 3.0) and acetonitrile

as mobile phase which was delivered initially in the ratio of 80:20 (v/v) for 18 min, then changed to 60:40 (v/v) for next 12 min, and finally equilibrated back to 80:20 (v/v) for 10 min. Other HPLC parameters were flow rate at 1.0 ml/min, detection wavelengths 248 and 280 nm, injection volume 10 ml. The calibration graphs plotted with five concentrations of each component were linear with a regression coefficient R2 >0.9999. The limit of detection and limit of quantitation were estimated for all the five impurities. The established method was then validated for linearity, precision, accuracy, and specificity and demonstrated to be applicable to the determination of the active ingredients in commercial and model cough syrup. No interference from the formulation excipients was observed. These results suggest that this LC method can be used for the determination of multiple active ingredients and their impurities in a cough and cold syrup.

Varaprasad Bobbarala *et al.*,[28] have developed a novel and single RP-HPLC method was developed for the determination of ten active ingredients (Codeine phosphate, Paracetamol, Chloropheniramine Maleate, Theophylline,Pseudoepidrine HCl, Ambroxol, Salbutamol, Guaiphenesin, Dextromethorphan and Diphenhydramine HCl) in all pharmaceutical dosage forms, along with preservative (Sodiumbenzoate) and validated the method as per ICH and FDA guidelines. The separation was achieved on a X-Terra C18, 15cm x 4.6mm, 3.5 2 in the simple gradient mode using Sol-A:buffer and Sol-B: Acetonitrile (0-5min, sol-A:97-97; 5-10min- sol-A:97-92; 10-15min- sol-A:92-68; 15-23min- sol-A:68-68; 23-25min- sol-A:68-97 and 25-30min- sol-A:97-97) with 0.8 mL per min flow rate. Column oven temperature maintained at 40°C and performed the analysis with 220 nm. Quantification was achieved with 402g per mL for all ingredients with 100 ±3.0% recovery. The method was validated by determining its sensitivity, linearity, accuracy and precision. The proposed method is single, shorter runtime, accurate and reproducible. This method can be applied for routine analysis of all ten active ingredients quantification in all pharmaceutical dosage forms.

Sandhya N Kumar *et al.*,[29] have developed Salbutamol Sulphate and bromhexine hydrochloride as components of a multi-ingredient formulation is useful in asthma therapy. This article reviews the Chromatographic methods for simultaneous determination of bromhexine and salbutamol in pharmaceutical samples. The most commonly adopted methods for the

determination of bromhexine and salbutamol are RP- HPLC. Hibar RP-C18 (250mm × 4.6mm, 5µm) column was used for separation, flow rate 1.2mL/min and wavelength was 220nm. Recent preferences in the simultaneous estimation of bromhexine and salbutamol proves primacy of RP- HPLC and confirms a general trend of moving towards more sensitive methods having higher resolution potential, consuming small quantities of samples and reagents and requiring less time for analysis.

Senthil raja M And GiriRaj P *et al.*,[30] have developed a simple, accurate and precise reversed phase HPLC method for rapid and simultaneous quantification of Terbutaline Sulphate, Bromhexine Hcl and Guaifenesin in a cough syrup formulation. Separations were carried out on a phenomenex Luna C18 column (250 X4.6 mm ID), 5 µm particle size. A isocratic elution system was developed using acetonitrile-methoanol-buffer (350:450:250 v/v). The elution of the analytes was achieved in less than 15 min with a flow rate of 1.2 ml/min. Detection was by UV absorbance at a wavelength of 220 nm. Quantification of the components in actual syrup formulations was calculated against the responses of freshly prepared external standard solutions. Different analytical performance parameters such as linearity, precision, accuracy, limit of detection, limit of quantification, and robustness were determined according to international conference on harmonization ICH Q2B guidelines. All the parameters of validation were found in the acceptance range of ICH guideline.

Raval Kashyap *et al.*,[31] have developed and validate a simple& accurate Spectrophotometry methods for simultaneous estimation of Ketotifen and Salbutamol in their combined pharmaceutical dosage form. Two simple, accurate, precise U.V Spectroscopy methods have been developed. First method was based on Simultaneous Equation method. Here 301 nm was selected for the estimation of Ketotifen& 276 nm selected for estimation of Salbutamol. The second method was Dual wavelength method, Here 284 nm & 267.84 nm selected for the estimation of Ketotifen where Salbutamol show same absorbance. Other 315 nm & 284.59 nm selected for estimation of Salbutamol where Ketotifen show same absorbance. Ketotifen and Salbutamol showed linearity in the range of 5-25µg/ml and 10- 50µg/ml respectively in both methods. Both methods were validated by validation parameters and it show result where lie within its acceptance criteria as per ICH Q2 (R1) guideline. Hence, it can be successfully used

for the routine analysis of Ketotifen and Salbutamolin their combined pharmaceutical dosage forms.

Rajan V. Rele *et al.*,[32] have developed A simple, rapid and accurate high performance liquid chromatography method is described for simultaneous determination of guaiphenesin and salbutamol sulphate from active pharmaceutical ingredients. The separation of drug was achieved on Zorbax Eclipse C18 (250 x 4.6 mm i.d.) with 5 µ particle size column showed most favorable chromatographic pattern over the other columns. The mobile phase consisted of a mixture of buffer of pH 4.3 and acetonitrile [75:25 % (v/v)]. The detection was carried out at wavelength 225 nm. The mixture of buffer of pH 4.3 and acetonitrile [75:25% (v/v)] was used as a diluent. The method was validated for system suitability, linearity,accuracy, precision, robustness, stability of sample solution. The method has been successfully used to analyze guaiphenesin and salbutamol sulphate from combined dosage form.

M. Abdelkawy *et al.*,[33] have developed the simultaneous determination of a mixture of Ambroxol HCl (A) and Guaifenesin (G) in presence of the oxidative degradate (AD) of A and guaicol (GD), the impurity of G . The first method is an isocratic HPLC method, where separation of the four components A, AD, G and GD was achieved on C18 column using water: methanol (80: 20, v/v, containing 1% triethylamine, pH 2.9) with a flow rate of 1.5 ml/min and UV detection at 220nm. A linear relationship in the range of 5-80 µg.ml-1 and 10-150 µg.ml-1 was obtained for A and G, respectively. The second method was a TLC-spectrodensitometric method, where the drugs together with AD and GD were applied on silica gel 60F254 plates and a mobile phase consisted of chloroform: methanol: ethyl acetate:acetic acid (70: 8: 12:10, by volume) was used for separation. Densitometric evaluation of the separated zones was performed at 270 nm. The regression analysis for the calibration plots showed good correlation over the range 1-7 µg/band and 2-10 µg/band for A and G, respectively. The third method was a multivariate spectrophotometric calibration method where principal component regression (PCR) and partial least squares (PLS) methods were used for the determination of the four components A, AD, G, and GD. The three methods were applied to pharmaceutical dosage forms containing either ambroxol alone (drops, capsules and tablets) or A together with G (syrup). Model update of multivariate calibration was used to determine A and G in syrup

dosage form due to interfering additives. Results for the three methods were statistically compared with those obtained by applying reference reported methods for the drugs and showed that the proposed methods are accurate, precise, and can be easily applied.

Nidhi dubey *et al.*,[34] have developed Simultaneous Determination of Ambroxol, Guaifenesin and Salbutamol in single dosage form .The method has been validated as per the guidelines of ICH and FDA .The finalized Reverse Phase-HPLC method is revealed with significant shorter retention time of 15 min with simple isocratic program .The separation is achieved on C-8,5µm;250mm× 4.6mm column with flow rate 1.0 ml, per minute in isocratic mode using disodium hydrogen ortho – phosphate buffer (4.5) and methanol as mobile phase column oven temperature is maintained at 25°C and observations are recorded at 220nm. The method is simple, accurate, reproducible and short and can be used for simultaneous analysis of ambroxol, guaifenesin and salbutamol in several single dose form formulation available in the market.

Paul M. Njaria *et al.*,[35] have developed for the Simultaneous Determination of Bromhexine, Guaifenesin, Ambroxol, Salbutamol/Terbutaline, Pseudoephedrine, Triprolidine and Chlorpheniramine maleate in cough –cold syrups commonly marketed in Kenya. Separation was achieved using a Gemini NX C_{18} column (250× 4.6mm,5µm) maintained at 40°C and a mobile phase consisting of acetonitrile -0.25 M ammonium acetate and pH 3.0-water (35:4:10:51,%v/v/v/v) delivered at 1.0 ml min^{-1}.The eluents were monitored by means of UV detection at 254nm .During validation ,the method satisfied the Inetrnational Committee On Hormonisation acceptance createria for linearity,sensitivity,precision,accuracy and robustness.The development liquid chromatographic method was applied in the analysis of nine commercial samples obtained from Nairobi city country,Kenya.Extraction procedure were not applied during the assay of the samples,the significantly shorting the analysis the time.

Srinivas Sumanth Kamatham *et al.*,[36] have developed method for simultaneous estimation of salbutamol (SAL), guaifenisin (GUA) and ambroxol (AMB) by high performance liquid chromatography (HPLC) in formulation was developed. The analysis was performed with a mobile phase containing acetonitrile (ACN) and potassium di-hydrogen phosphate (PDHP) adjusted to a pH- 4 in the ratio of 70 : 30 (% v/v) at a flow rate of 1.0 ml/min. A SHISEIDO C18 column (250 x 4.6mm i.d; 5µm) was used. A UV spectrum of salbutamol, guaifenisin and

ambroxol was recorded by scanning between 200-400 nm, from the overlapping spectra a wavelength of 215 nm is selected and simultaneous estimation is carried out. The analysis was performed in the linearity range of 2-16 µg/ml for Salbutamol, 0.5-4 µg/ml for Ambroxol and 5-40 µg/ml for Guaifenisin respectively. The correlation coefficient was 0.996 (SAL), 0.994 (AMB), 0.998 (GUA). Thus the developed and validated chromatographic method for Salbutamol, Ambroxol and Guaifenisin is said to be rapid, precise (RSD<=2%), simple, accurate (% recovery=90-101%).

Nirav C. Patel *et al.*,[37] have developed a method for estimation of Ambroxol HCl, Guaifenesin and Levosalbutamol sulphate in syrup form. The study was done by combining three spectrophotometric methods viz use of first order derivative and colorimetry. The separation of Analytes was achieved within 10 min, employing a mixture of 10Mm triethylamine-phosphoric acid buffer (pH 3.0) and MeOH (60:40,%v/v) as isocratic mobile phase , pumped at 1.0ml min throgh a cyano column(5lm partical size). The analytes were detected at 215nm. Absorption of Guaifenesin and Levosalbutamol sulphate were found to be zero at 323 nm, thus enabling the measurement of Ambroxol HCl, using specific absorbance in first order spectrum. Same way absorption of Ambroxol HCl and Levosalbutamol sulphate were found to be zero at 276 nm, thus enabling the measurement of Guaifenesin using specific absorbance in first order spectrum. For colorimetric measurement of Levosalbutamol Sulphate, a colored substance was obtained by coupling the oxidized product of Levosalbutamol sulphate with 4-aminoantipyrine and Potassium ferricyanide and its absorption was measured at 503 nm. The proposed method was statistically validated in accordance with ICH guidelines and results were found to be satisfactory for accuracy, precision and specificity.

Levon A.Melikyan *et al.*,[38] have developed and validated for quantitative determination of Guaifenesin impurities including 2-(2- methoxyphenoxy)propane-1,3-diol(β-isomer) and 2-methoxyphenoxy (guaicol) in different multi drug components pharmaceutical dosage forms,containing guaifenesin, ambroxol hydrochloride and salbutamol sulfate.The different analytical performance parameters such as linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) were determined according to International Conference on Hormonization(ICH) Q2B guidelines.The chromatographic separation was achieved on EC NUCLEODUR-100-3C18(250 ×4.6mm,5µm packing) column using gradient elution of solvent

A (0.1M ammonium acetate buffer of pH 6.8) and solvent B (acetonitrile : methanol (80:20)). The Ultra Violet Spectrophotometric determination was performed at 275nm.The linearity of the calibration curves for the analytes in the desired concentration range is good (r2=0.999) by High Performance Liquid Chromatography.The LOQ were 1 and 0.1µg/ml respectively for guaifenesin β-isomer and guaiacol.The percentage recovery of guaifenesin impurities was found to be within 98.6-101.2% of range.The developed method can be successfully used for identification and quantification of guaifenesin impurities β-isomer and guaiacol in the presence of guaifenesin ,ambroxol hydrochloride and salbutamol sulfate in multi drug components pharmaceutical formulations.

Silvana E. Vignaduzzo *et al.*,[39] have developed and validated for the Simultaneous determination of bromhexine, chlorpheniramine, paracetamol, and pseudoephedrine in common cold medications (tablets and syrups).The separation of Analytes was achieved within 10 min, employing a mixture of 10Mm triethylamine-phosphoric acid buffer (pH 4.0) and MeOH (35:65,v=v) as isocratic mobile phase , pumped at 1.0ml min through a cyano column(5lm partical size).The analytes were detected at 215nm. Statistical experimental designs and graphic representations (response surface methodologies, Pareto charts)were used for selecting the proper detection wavelength, optimizing the mobile phase composition, and assessing method robustness. The linearity of the calibration($r>0.99, n^{1/4}21$) in the relevant ranges (up to 130% of the expected concentrations of the analytes in the formulation), method accuracy (bias<2.0%), repeatability (RSD<2.0%) and intermediate precision, were verified. In addition, specificity (peak purities with photodiode array detector >0.9997) and method robustness were evaluated, and system suitability parameters were determined .The validated method was successfully employed for the routine analysis of various commercial tablet syrup pharmaceutical against the common cold, showing satisfactory analyte recoveries and RSD values.

4. AIM AND PLAN OF WORK

The extensive literature survey carried out revealed few methods have been reported for simultaneous estimation of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride and in combination with other drugs. However there is no method reported for the simultaneous estimation of Salbutamol, Guaifenesin and Ambroxol hydrochloride in oral liquid dosage form. So it was felt that there is a need to develop RP-HPLC method for the determination of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride simultaneously in single step process. Hence the present work is aimed to develop reverse phase HPLC method for the simultaneous determination of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride in oral liquid dosage form and validation of the developed method.

The plan of the work for the aimed study was designed as follows:

- Method development by RP-HPLC
    - Selection of suitable wavelength
    - Selection of stationary phase
    - Selection of mobile phase
    - Optimization of chromatographic conditions

- Validation of developed method using the following parameters
    - System suitability
    - Accuracy
    - Precision
        - System precision
        - Method precision
        - Intermediate precision
    - Linearity
    - Robustness
    - Specificity
    - Solution stability

5. MATERIALS AND METHODS

Drug samples

Salbutamol sulphate, Guaifenesin and Ambroxol hydrocloride raw materials were obtained as gift samples from Bioplus Life Sciences Pvt, Ltd, Hosur.

Formulation used

CRMS Syrup (100mL) containing 50 mg/5mL of Guaifenesin, 15 mg/5mL of Ambroxol and 1 mg/5mL of Salbutamol, manufactured by Pharmed Ltd., Whitefield road, Bangalore was procured from local market.

Instruments used

1) HPLC - agilent / chemstation
2) Agilent separation module 1200
3) Injector - Auto Injector
4) Column - Inertsil C_8-3 (250 x 4.6 mm, 5 µ)
5) Detector - Agilent DAD or UV detector
6) pH meter - Adwa AD 1020
7) Jasco - Double beam UV-VISIBLE spectrophotometer.
8) Denver - Analytical balance (10 mg – 200 gm)
9) Hot Air Oven - Techno Lab, model BTZ

Reagents and Chemicals

1) Sodium dihydrogen phosphate (AR grade)
2) Triethyl amine(LR grade)
3) Ortho-phosphoric acid (AR grade)
4) Acetonitrile (HPLC grade)
5) Methanol (HPLC Grade)
6) Water (Milli Q)

Reference Standards

a. **Guaifenesin**

% Purity –99.7% w/w

b. **Ambroxol hydrochloride**

% Purity –99.8% w/w

c. **Salbutamol sulphate**

% Purity –99.8% w/w

6. RESULTS AND ANALYSIS

6.1. DEVELPOMENT AND OPTIMIZATION OF CHROMATOGRAPHIC PARAMETERS

6.1.1. Solubility

According to literature review, Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride are freely soluble in methanol. Therefore the solubility of the drugs was checked with different dilutions of phosphate buffer (pH-3), methanol and acetonitrile. Finally buffer: acetonitrile: methanol in the ratio 65:10:25 was chosen as solvent for present work.

6.1.1.2. Selection of chromatographic method

The choice of the chromatographic method is based on the nature of the sample (ionic or neutral molecule), its molecular weight and solubility. As drugs are polar in nature, the reverse phase chromatographic technique was selected for the present study.

6.1.1.3. Selection of wavelength (λmax)

In setting up the conditions for development of the assay method, the choice of detection wavelength was based on the scanned absorption spectrum for Salbutamol, Ambroxol and Guaifenesin. The UV- Spectrum of Salbutamol, Ambroxol and Guaifenesin were obtained separately.

Figure 3 UV-Spectrum of Salbutamol sulphate

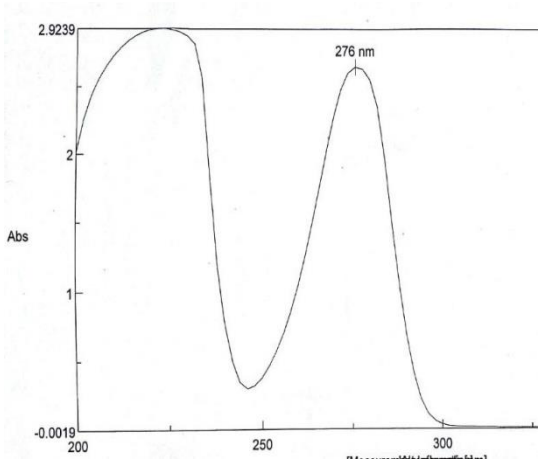


Figure 4 UV-Spectrum of Guaifenesin

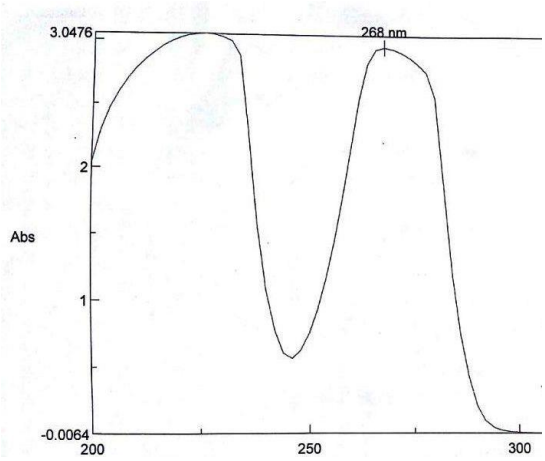
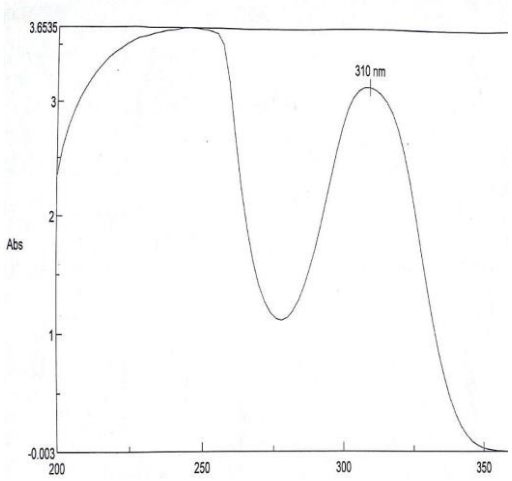


Figure 5 UV-Spectrum of Ambroxol hydrochloride



By scanning the sample solution Salbutamol, Ambroxol and Guaifenesin each 10µg/mL in methanol over the wavelength range 200-400 nm against blank. After thorough examination of the spectra, the wavelength 276 nm was chosen for further analysis.

6.2 METHOD DEVELOPMENT TRIALS

6.2.1. Sample preparation

Salbutamol, Ambroxol and Guaifenesin each 10µg/mL solution in methanol was prepared and used for trials.

Trial – 1

The trial 1 was performed with Water: Methanol in the ratio of 50:50%v/v with flow rate 1 mL/min.

Only two peaks were identified.

Trial – 2

The trial 2 was performed with Water: Methanol: Acetonitrile in the ratio of 40:30:30%v/v with flow rate 1 mL/min.

Only two peaks were obtained.

In Water: Methanol and water: Acetonitrile only two peaks were identified. So, instead of water, next trails were performed with Sodium dihydrogen Phosphate buffer (pH-3.0).

Trial – 3

The trial 3 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 50:25:25%v/v with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Salbutamol and Guaifenesin.

Trial – 4

The trial 4 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 80:10:10%v/v with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Guaifenesin and Ambroxol.

Trial – 5

The trial 5 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 40:30:30%v/v with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Salbutamol and Guaifenesin.

Trial – 6

The trial 6 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 60:5:30%v/v with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Salbutamol and Guaifenesin.

Trial – 7

The trial 7 was performed by using Buffer: Acetonitrile: Methanol in the gradient method 0-5 mins buffer 100%, 5.1-10 mins buffer 75%: Acetonitrile 15%: Methanol 15%, 10.1-15 mins buffer 100% with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Guaifenesin and Ambroxol.

Trial – 8

The trial 8 was performed by using Buffer: Acetonitrile: Methanol in the gradient method 0-4 mins buffer 100%, 4.1-15 mins buffer 65%: Acetonitrile 15%: Methanol 20%, 15.1-18 mins buffer 100% with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Guaifenesin and Ambroxol.

Trial – 9

The trial 9 was performed by using Buffer: Acetonitrile: Methanol in the gradient method 0-10 mins buffer 100%, 10.1-22 mins buffer 65%: Acetonitrile 15%: Methanol 20%, 22.1-25 mins buffer 100% with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Guaifenesin and Ambroxol.

Trial – 10

The trial 10 was performed by using Buffer: Acetonitrile: Methanol in the gradient method 0 mins buffer 90%: Acetonitrile 5%: Methanol 5%, 10 mins buffer 86%: Acetonitrile 7%: Methanol 7%, 14 mins buffer 70% : Acetonitrile 15%: Methanol 15%, 25 mins buffer 65%: Acetonitrile 15%: Methanol 20%, 28-30 mins buffer 90%: Acetonitrile 5%: Methanol 5%, with flow rate 1 mL/min.

Only two peaks were eluted (i.e) Guaifenesin and Ambroxol.

Trial – 11

The trial 11 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 75:20:5%v/v with flow rate 1 mL/min.

Interference in Salbutamol and Ambroxol.

Trial – 12

The trial 12 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 70:15:15%v/v with flow rate 1 mL/min.

Interference in Salbutamol and Ambroxol.

Trial – 13

The trial 13 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 65:15:20%v/v with flow rate 1 mL/min.

The retention time of Salbutamol was found to be 3.1, Guaifenesin was eluted in 6.1 and Ambroxol was eluted in 9.8. Small hump on Salbutamol peak .

Trial – 14

The trial 14 was performed by using Buffer: Acetonitrile: Methanol in the ratio of 65:10:25%v/v with flow rate 1 mL/min.

The retention time of Salbutamol, Guaifenesin and Ambroxol was found to be 3.2, 9.8 and 11.8mins respectively.

Conclusion

Out of the 14 trials, the 14^{th} trial was selected for further studies. When compared to the other trials the in the 14^{th} trial has good plate count, Tailing factor, Symmetry and Resolution observed and peak shape was also good.

6.3 OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Stationary Phase	:	Inertsil C8-3 (250 mmx4.6 mm , 5 µ)
Separation module	:	Agilent 1200
Injector	:	Auto injector
Flow rate	:	1.0 mL/min
Operating temperature	:	Ambient
Selected wave length	:	276 nm
Mobile phase ratio	:	Buffer: Acetonitrile: Methanol (65:10:25 %v/v)
Diluents	:	Mobile Phase
Injection Volume	:	10 µL
Run Time	:	25 min

6.4 PREPARATION OF SOLUTIONS

6.4.1. Preparation of Phosphate buffer

Weighed 1.56 grams of NaH_2PO_4 into a 1000ml beaker, dissolved and to 1000 mL with water, add 3 mL of TEA and adjusted the pH to 3.0 with Ortho-phosporic acid.

6.4.2. Preparation of mobile phase

Mix a mixture of above buffer 650 mL (65%), 100 mL of Acetonitrile (10%) and 250 mL of Methanol (25%) degassed in ultrasonic water bath for 5 minutes. Filtered through 0.45µ filter under vacuum filtration.

6.4.3. Standard solution

Preparation of Salbutamol, Ambroxol and Guaifenesin stock solution:

Accurately weighed quantity of 500 mg of Guaifenesin, 150 mg Ambroxol and 25 mg of Salbutamol was transferred to three different 100 mL volumetric flask, dissolved in 25 mL of mobile phase, sonicated for 5 min and the volume was made up with mobile phase.

6.4.4. Concentration of stock solution

Guaifenesin	-	500 µg/mL
Ambroxol hydrochloride	-	150 µg/mL
Salbutamol sulphate	-	10 µg/mL

6.4.5. Preparation of working standard

Working standard for Guaifenesin, Ambroxol and Salbutamol were prepared by pipetting 10 mL, 10mL and 4 mL respectively from each of the stock solution in a 100 mL

volumetric flasks and the volume was made up with the mobile phase to give the following concentration.

Guaifenesin	-	500 µg/mL
Ambroxol hydrochloride	-	150 µg/mL
Salbutamol sulphate	-	10 µg/mL

6.4.6. Sample preparation

Accurately measure, amount equivalent to 500 mg of Guaifenesin, 150 mg of Ambroxol and 25 mg of salbutamol from liquid formulation was accurately weighed and taken in three different 100 mL volumetric flask and 25 mL of mobile phase was added. The mixture was subjected to sonication for 15 min with intermediate shaking for complete dissolving of drugs. Cooled to room temperature and the solution was made up to the mark with mobile phase. Filtered using 0.45µ filter. Then 10 µL of this solution was injected for HPLC analysis.

6.4.7. Procedure

Separately blank, standard and test solutions were injected and the areas for major peaks were recorded for Guaifenesin, Ambroxol and Salbutamol and % assay was calculated by using the following formula.

$$\frac{AT \times WS \times DT \times P \times \text{Avg. Wt}}{AS \times DS \times WT \times 100 \times \text{Label Claim}} \times 100$$

Where:

AT = average area counts of sample preparation.

As = average area counts of standard preparation.

WS = Weight of working standard taken in mg.

WT = Weight of test taken in mg.

DS = Standard dilution

DT = Test dilution

P = Percentage purity of working standard

LC = label claim mg/mL.

6.5. METHOD VALIDATION

6.5.1. Introduction:

The method has been validated for Salbutamol sulphate, guaifenesin and ambroxol hydrochloride (CRMS Syrup) by following validation parameters,

The method validation involves establishing of the following parameters

a. System suitability
b. Accuracy
c. Precision
    i. System Precision/Repeatability
    ii. Method Precision
    iii. Intermediate Precision/Ruggedness
d. Linearity
e. Robustness
f. Specificity
g. Solution stability

6.5.2. System suitability studies

Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride solutions were prepared and injected. Then the system suitability parameters like Resolution, Retention Time, plate number (N), Peak asymmetry factor (Tailing) were evaluated with the help of standard chromatogram.

Table 4 Results for System suitability Parameters

Parameters	Salbutamol sulphate	Guaifenesin	Ambroxol hydrochloride	Acceptance criteria
Resolution	NA	29.90	4.69	NLT 2
Tailing factor	1.1	1.1	1.3	NLT 2
Number of theoretical plate	14133	13119	9978	NLT 2000
Retention time	3.157	9.949	11.883	NA

6.5.3. Accuracy

Accuracy expresses the closeness of agreement between the value, which is accepted either as conventional true value or and accepted reference value (International Standard e.g.

Pharmacopoeia Standard) and the value found (mean value) obtained by applying the test procedure a number of times.

The recovery study was carried out at 50%, 100% and 150% level and the contents were determined from the respective chromatogram. From the results obtained we can conclude that the method was accurate

Table 5 Results for Recovery Study for Salbutamol

Accuracy Level	Amount of Salbutamol Added (µg)	Amount of Salbutamol Found (µg)	% Recovery	% Mean Recovery	SD	% RSD
Accuracy solution 50%-1	4.99	4.91	101.6	101.5	0.23	0.23
Accuracy solution 50%-2		4.93	101.2			
Accuracy solution 50%-3		4.91	101.6			
Accuracy solution 100%-1	9.98	9.87	101.1	101.3	0.15	0.15
Accuracy solution 100%-2		9.85	101.3			
Accuracy solution 100%-3		9.84	101.4			
Accuracy solution 150%-1	14.97	14.78	101.3	101.2	0.15	0.15
Accuracy solution 150%-2		14.82	101.0			
Accuracy solution 150%-3		14.79	101.2			

Acceptance Criteria: The % Recovery for each level should be between 98.0% to 102.0%.

Table 6 Results for Recovery Study for Guaifenesin

Accuracy Level	Amount of Guaifenesin Added (µg)	Amount of Guaifenesin Found (µg)	% Recovery	% Mean Recovery	SD	% RSD
Accuracy solution 50%-1	249.25	252.65	98.7	98.7	0.15	0.15
Accuracy solution 50%-2		253.12	98.5			
Accuracy solution 50%-3		252.16	98.8			
Accuracy solution 100%-1	498.50	504.58	98.7	98.5	0.20	0.20
Accuracy solution 100%-2		505.85	98.5			
Accuracy solution 100%-3		507.25	93.3			
Accuracy solution 150%-1	747.75	758.12	98.6	98.4	0.21	0.21
Accuracy solution 150%-2		760.85	98.3			
Accuracy solution 150%-3		761.65	98.2			

Acceptance Criteria: The % Recovery for each level should be between 98.0% to 102.0%.

Table 7 Results for Recovery Study for Ambroxol

Accuracy Level	Amount of Ambroxol hydrochloride Added (µg)	Amount of Ambroxol hydrochloride Found (µg)	% Recovery	% Mean Recovery	SD	% RSD
Accuracy solution 50%-1	74.85	74.15	100.6	100.5	0.25	0.25
Accuracy solution 50%-2		73.89	100.9			
Accuracy solution 50%-3		74.25	100.4			
Accuracy solution 100%-1	149.70	148.42	100.8	100.6	0.26	0.26
Accuracy solution 100%-2		148.62	100.7			
Accuracy solution 100%-3		149.12	100.3			
Accuracy solution 150%-1	224.55	222.58	100.9	100.7	0.15	0.15
Accuracy solution 150%-2		222.96	100.7			
Accuracy solution 150%-3		223.16	100.6			

Acceptance Criteria: The % Recovery for each level should be between 98.0% to 102.0%.

6.5.4. Precision

6.5.4.1. System Precision/Repeatability of Injection

The **system precision** was performed by injecting standard solution for five times on to the analytical column and the peak area was measured then %RSD for the area of five replicate injections was calculated.

Table 8 Results for System Precision of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride

S. No	Area of Salbutamol sulphate	Area of Guaifenesin	Area of Ambroxol hydrochloride
1	38.112	3488.011	214.053
2	38.998	3498.539	216.653
3	38.620	3507.698	214.790
4	38.148	3506.391	215.290
5	38.613	3510.320	216.647
Mean	38.498	3502.012	215.491
S.D	0.37	9.06	1.15
%RSD	1.0	0.3	0.5

Acceptance Criteria: The %R SD should be NMT 2.0%

6.5.4.2. Method Precision

The **method precision** was done by performing assay on six replicate determination of sample preparation at test concentration level (as per method of analysis) and the relative standard deviation of assay results was obtained.

Table 9 Results for Method Precision of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride

S.No	Area of Salbutamol sulphate	% Assay	Area of Guaifenesin	% Assay	Area of Ambroxol hydrochloride	%Assay
1	38.011	101.86	3556.132	98.43	214.985	100.28
2	38.108	101.56	3552.128	98.54	214.856	100.34
3	38.214	101.31	3553.563	98.49	214.988	100.28
4	38.256	101.20	3557.256	98.39	215.011	100.27
5	38.302	101.08	3554.982	98.46	215.141	100.21
6	38.359	100.93	3557.211	98.39	215.210	100.18
Mean	38.211	101.32	3555.212	98.45	215.032	100.26
SD	0.13	0.34	2.06	0.06	0.12	0.06
%RSD	0.33	0.33	0.06	0.06	0.06	0.06

Acceptance Criteria: The %RSD should be NMT 2.0%

6.5.4.3. Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Table 10 Results for Intermediate Precision of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride

S.No	Area of Salbutamol sulphate	% Assay	Area of Guaifenesin	% Assay	Area of Ambroxol hydrochloride	%Assay
1	38.154	101.47	3561.012	98.29	214.654	100.44
2	38.210	101.33	3563.254	98.23	214.852	100.35
3	38.245	101.23	3568.453	98.09	214.741	100.39
4	38.311	101.06	3559.245	98.34	214.998	100.28
5	38.345	100.97	3560.854	98.29	215.014	100.27
6	38.352	100.95	3562.852	98.24	214.961	100.29
Mean	38.269	101.17	3562.61	98.25	214.870	100.34
SD	0.08	0.21	3.21	0.09	0.15	0.07
%RSD	0.21	0.31	0.09	0.09	0.07	0.07

Acceptance Criteria: The %RSD should be NMT 2.0

6.5.5. Linearity and Range

6.5.5.1. Preparation of Level – I

5mL, 5mL and 2mL of stock solution for Salbutamol, Guaifenesin and Ambroxol were taken in 100mL of volumetric flask diluted up to the mark with mobile phase.

6.5.5.2. Preparation of Level – II

7.5mL, 7.5mL and 3mL of stock solution for Salbutamol, Guaifenesin and Ambroxol were taken in 100mL of volumetric flask diluted up to the mark with mobile phase.

6.5.5.3. Preparation of Level – III

10mL, 10mL and 4mL of stock solution for Salbutamol, Guaifenesin and Ambroxol were taken in 10mL of volumetric flask diluted up to the mark with mobile phase.

6.5.5.4. Preparation of Level – IV

12.5mL, 12.5mL and 5mL of stock solution for Salbutamol, Guaifenesin and Ambroxol were taken in 10mL of volumetric flask diluted up to the mark with mobile phase.

6.5.5.5. Preparation of Level – V

15mL, 15ml and 6mL of stock solution for Salbutamol, Guaifenesin and Ambroxol were taken in 10mL of volumetric flask diluted up to the mark with mobile phase.

6.5.5.6. Procedure

Solution of each level was injected into the chromatographic system and the peak area was measured.

Salbutamol sulphate showed linearity in the range of 5-15 (µg/mL), Guaifenesin showed linearity in the range of 250-750 (µg/mL) and Ambroxol hydrochloride showed linearity in the range of 75-225 (µg/mL).The calibration graphs was plotted with peak area in the Y axis and concentration of standard solution in the X axis. The degree of linearity was estimated by calculating the correlation coefficient.

The correlation coefficient values for Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride were found to be respectively.

Table 11 Results for Linearity Data

Concentration of Salbutamol sulphate (µg/mL)	Peak Area	Concentration of Guaifenesin (µg/mL)	Peak Area	Concentration of Ambroxol hydrochloride (µg/ml)	Peak Area
05	20.298	250	1784.443	75	110.443
7.5	30.306	375	2676.681	112.5	166.149
10	39.608	500	3520.368	150	220.646
12.5	48.196	625	4422.826	187.5	272.420
15	57.828	750	5267.516	225	326.137
Correlation coefficient - 0.9994		Correlation coefficient - 0.9999		Correlation coefficient - 0.9998	

Acceptance Criteria: The correlation coefficient should be NLT 0.99

Figure6 Linearity Plot for Salbutamol sulphate

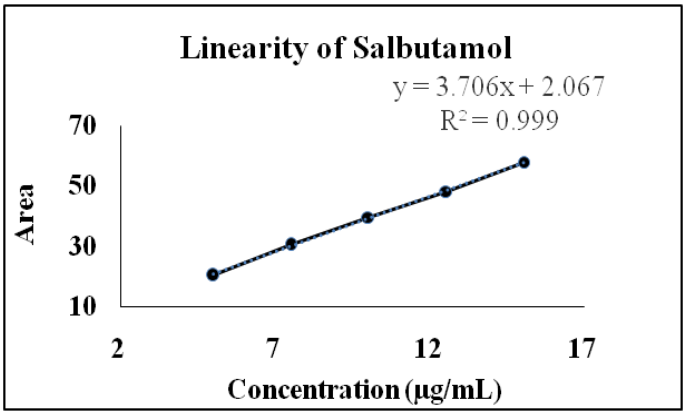


Figure 7 Linearity Plot for Guaifenesin

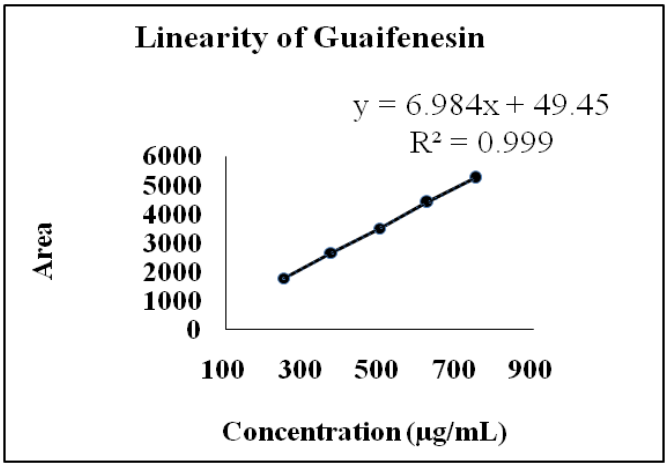
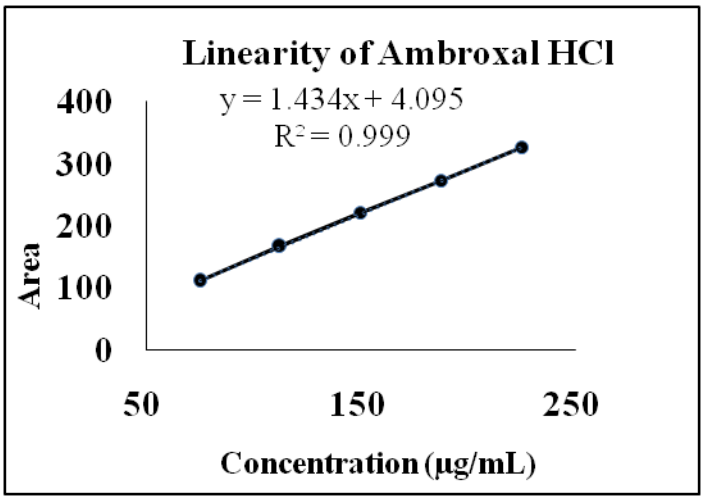


Figure 8 Linearity Plot for Ambroxol hydrochloride



6.5.6. Robustness

For demonstrating the robustness of the developed method, experimental conditions were purposely altered and evaluated. The method must be robust enough to withstand such slight changes and allow routine analysis of the sample. Following optimized conditions were slightly varied.

6.5.6.1. Effect of Flow rate

Robustness of assay method was carried out with variation of flow rate ±0.1mL/min of the set value i.e. 1mL/min. Standard solution was prepared and performed analysis as per test method and evaluated the system suitability parameters.

Table 12 Results for effect of Flow rate

Name	Flow rate mL/Min	RT	Plate count	Tailing	Resolution
Salbutamol sulphate	0.9	3.49	11482	0.8	NA
	1.0	3.15	14133	1.1	NA
	1.1	2.80	10403	0.8	NA
Guaifenesin	0.9	10.88	13848	1.1	29.52
	1.0	9.94	13119	1.1	29.90
	1.1	9.01	12690	1.1	28.52
Ambroxol hydrochloride	0.9	12.86	10421	1.3	4.52
	1.0	11.88	9978	1.3	4.69
	1.1	10.93	9477	1.3	4.48

Table 13 Results for change in flow rate (0.9 ml/Min)

S. No	Area of Salbutamol sulphate	Area of Guaifenesin	Area of Ambroxol hydrochloride
1	38.011	3488.011	214.053
2	38.108	3498.539	216.653
Mean	38.056	3493.275	215.353
S.D	0.07	7.44	1.84
%RSD	0.18	0.21	0.85

Acceptance Criteria : %RSD should be NMT 2

Table 14 Results for change in flow rate (1.0 mL)

S. No	Area of Salbutamol sulphate	Area of Guaifenesin	Area of Ambroxol hydrochloride
1	38.684	3506.425	215.243
2	38.298	3510.122	215.987
Mean	38.491	3508.274	215.615
S.D	0.27	2.61	0.54
%RSD	0.71	0.07	0.24

Acceptance Criteria : %RSD should be NMT 2

Table 15 Results for change in flow rate (1.1 mL/Min)

S. No	Area of Salbutamol sulphate	Area of Guaifenesin	Area of Ambroxol hydrochloride
1	37.854	3486.218	213.458
2	38.024	3488.452	213.853
Mean	37.939	3487.335	213.655
S.D	0.12	1.58	0.28
%RSD	0.32	0.05	0.13

Acceptance Criteria : %RSD should be NMT 2

6.5.6.2. Effect of Wavelength

Robustness of assay method was carried out with variation of wavelength ±2nm of the set value i.e. 276nm. Standard solution was prepared and performed analysis as per test method and evaluated the system suitability parameters.

Table 16 Results for effect of Wavelength

Name	Wavelength (nm)	RT	Plate count	Tailing	Resolution
Salbutamol sulphate	274	3.56	11882	0.8	NA
	276	3.15	14133	1.1	NA
	278	2.86	11403	0.8	NA
Guaifenesin	274	10.98	12848	1.1	29.22
	276	9.94	13119	1.1	29.90
	278	9.31	11690	1.1	28.98
Ambroxol hydrochloride	274	12.46	10521	1.3	4.82
	276	11.88	9978	1.3	4.69
	278	11.13	9677	1.3	4.28

Table 17 Results for change in wavelength (274nm)

S. No	Area of Salbutamol sulphate	Area of Guaifenesin	Area of Ambroxol hydrochloride
1	39.021	3504.258	216.687
2	39.254	3508.654	216.458
Mean	39.138	3506.456	216.573
S.D	0.16	3.11	0.16
%RSD	0.42	0.09	0.07

Acceptance Criteria : %RSD should be NMT 2

Table 18 Results for change in wavelength 276nm

S. No	Area of Salbutamol sulphate	Area of Guaifenesin	Area of Ambroxol hydrochloride
1	38.698	3498.254	215.124
2	38.386	3501.568	215.354
Mean	38.542	3499.911	215.239
S.D	0.22	2.34	0.16
%RSD	0.57	0.07	0.08

Acceptance Criteria : %RSD should be NMT 2

Table 19 Results for change in wavelength (278nm)

S. No	Area of Salbutamol sulphate	Area of Guaifenesin	Area of Ambroxol hydrochloride
1	38.054	3488.478	214.689
2	38.114	3492.854	214.287
Mean	38.084	3490.666	214.488
S.D	0.04	3.09	0.28
%RSD	0.11	0.09	0.13

Acceptance Criteria : %RSD should be NMT 2

6.5.7. Specificity

In case of simultaneous assay of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients.

This comparison should include samples stored under relevant stress conditions.

Table 20 Results for Peak purity

Name	Retention Time			Peak Purity
	Salbutamol sulphate	Guaifenesin	Ambroxol hydrochloride	
Blank	Not detected	Not detected	Not detected	NA
Placebo	Not detected	Not detected	Not detected	NA
Standard	3.13	9.99	11.88	1.1
Control Sample	3.14	9.95	11.86	1.1

Acceptance Criteria : Peak purity should be not less than 1

Table 21 Results for %Assay

Name	Lable claim (mg)	Amount found (mg)	% Assay
Salbutamol sulphate	1	1.01	101.00
Guaifenesin	50	49.68	99.36
Ambroxol hydrochloride	15	14.98	99.87

Acceptance Criteria : % Assay should be between 98% to 102%

6.5.9. Solution Stability

Standard solution was prepared as per the test method and stored at room temperature (23^0C-27^0C). Solution stability study to be performed at different days, against freshly prepared standard solution.

Table 22 Results for Standard solution stability

Interval	Room Temperature (23^0C-27^0C)					
	Salbutamol Area	Guaifenesin Area	Ambroxol Area	Similarity factor		
				Salbutamol	Guaifenesin	Ambroxol
Initial	38.620	3507.698	214.790	1.01	1.00	1.00
24 Hrs	37.557	3497.494	213.358	1.00	1.01	1.01
36 Hrs	38.998	3526.216	215.165	0.99	1.01	1.01
48 Hrs	40.356	3598.583	216.639	1.01	1.00	1.00

Acceptance Criteria: The similarity factor should be 0.98-1.02

Sample solution was prepared as per the test method and stored at room temperature (23^0C-27^0C). Solution stability study to be performed at different days, against freshly prepared Sample solution.

Table 23 Results for Sample solution stability

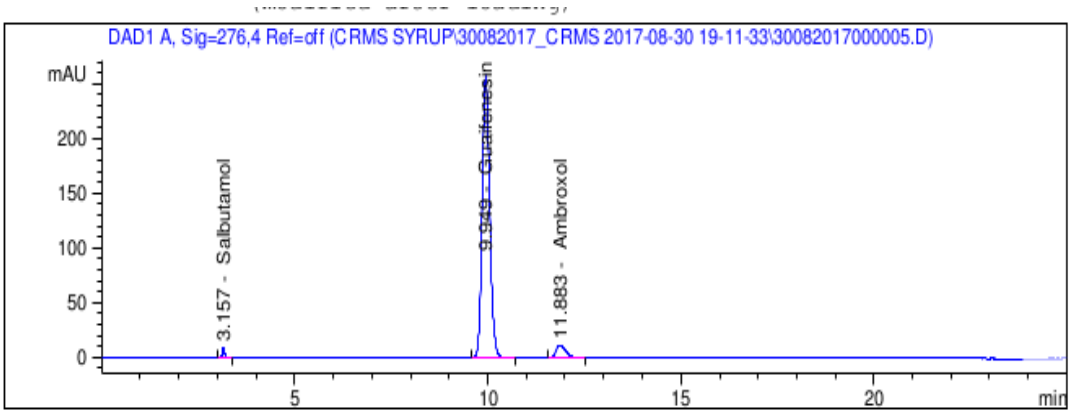
Interval	Room Temperature (23^0C-27^0C)								
	SAB Area	GUA Area	AMB Area	% Assay			Absolute % Difference		
				SAB	GUA	AMB	SAB	GUA	AMB
Initial	38.256	3557.256	215.011	101.58	99.56	100.88	NA	NA	NA
24 Hrs	38.557	3602.494	214.058	101.15	99.15	100.56	0.4	0.4	0.3
36 Hrs	39.987	3687.216	214.665	100.96	98.56	100.05	0.2	0.6	0.5
48 Hrs	40.998	3714.583	216.139	100.85	98.21	99.87	0.1	0.3	0.2

Acceptance Criteria: The absolute % difference should be NMT 1.0

7. CHROMATOGRAMS

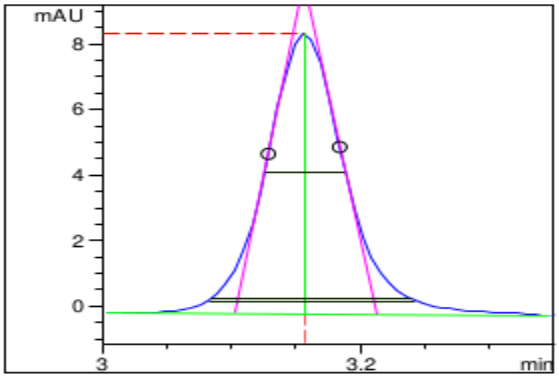
System suitability:

Plate Count

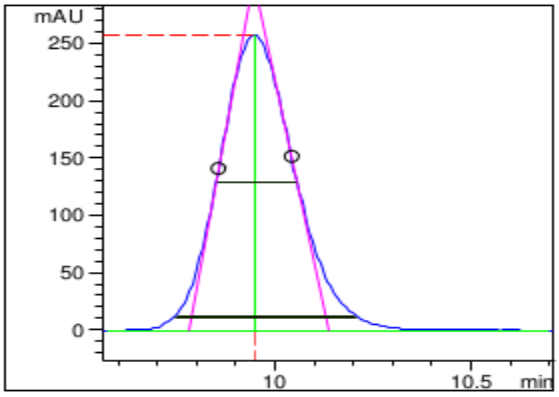


Tailing

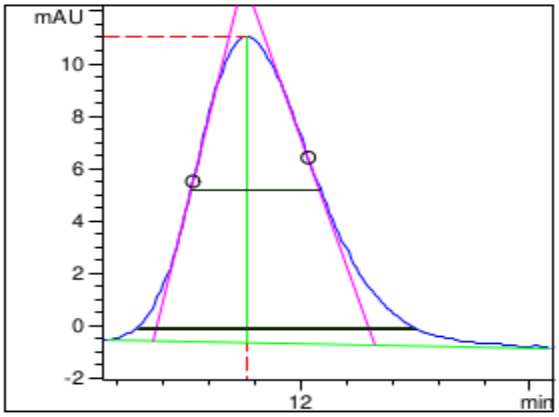
Salbutamol sulphate



Guaifenesin

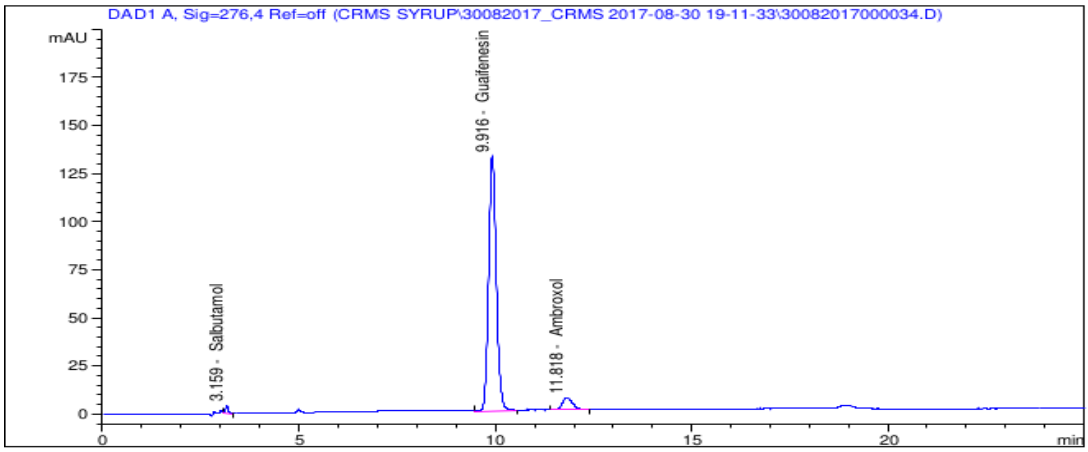


Ambroxol hydrochloride

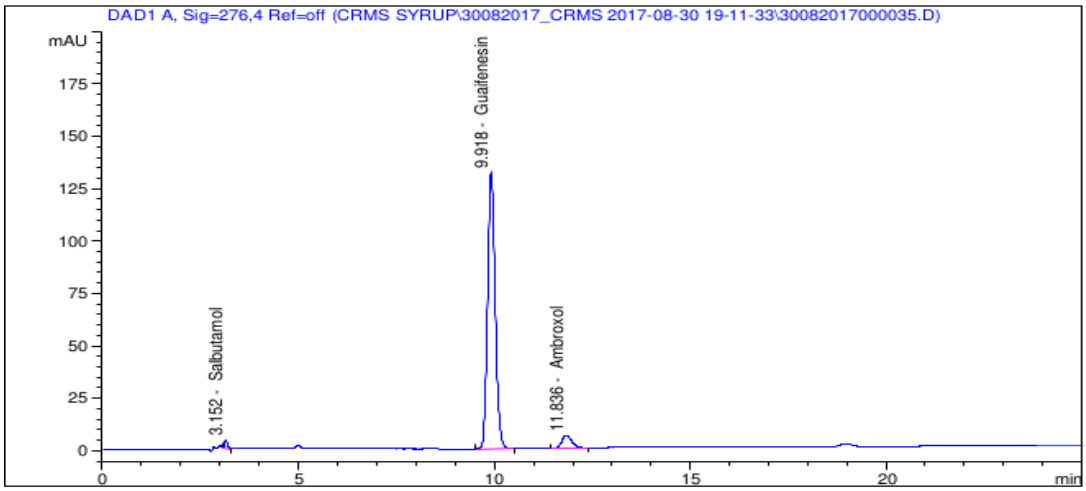


Accuracy

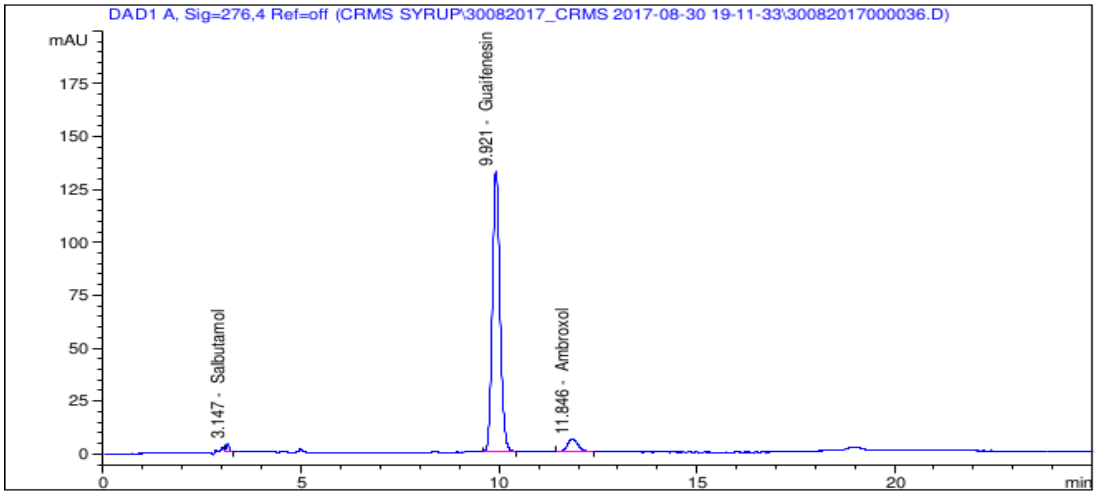
Level 50%-1_1



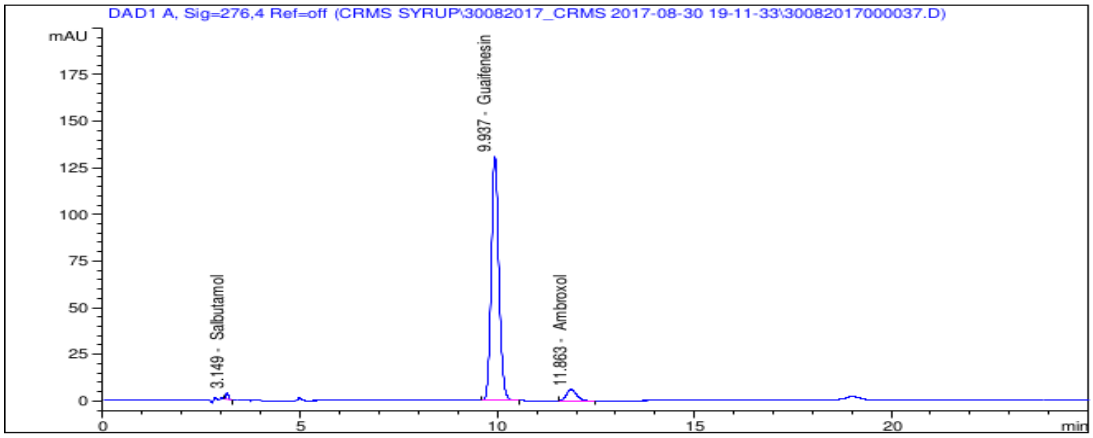
Level 50%-1_2



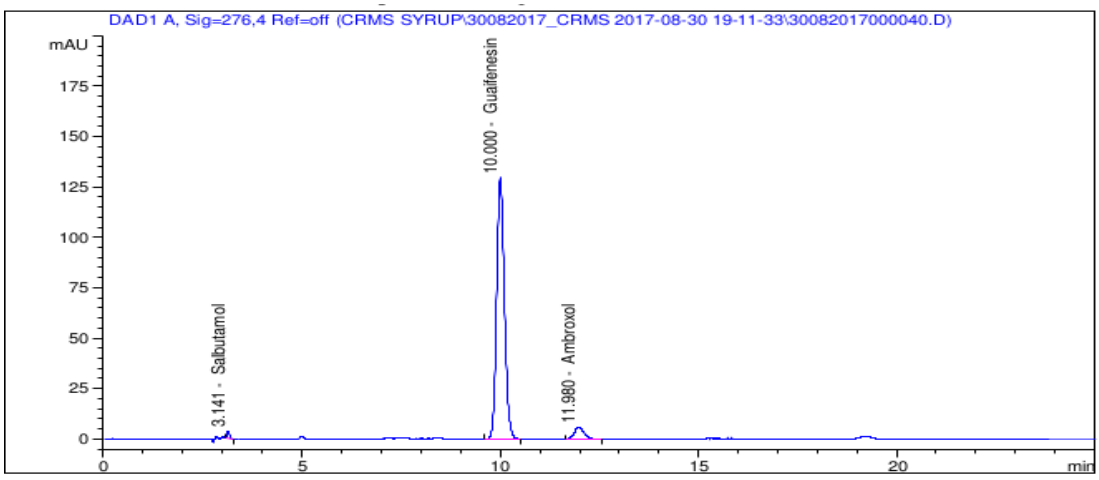
Level 50%-2_1



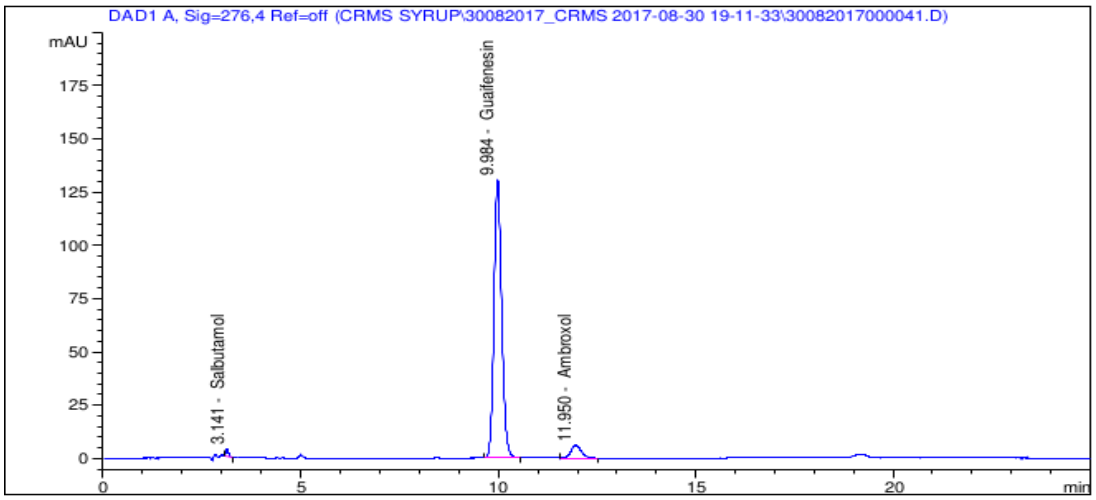
Level 50%-2_2



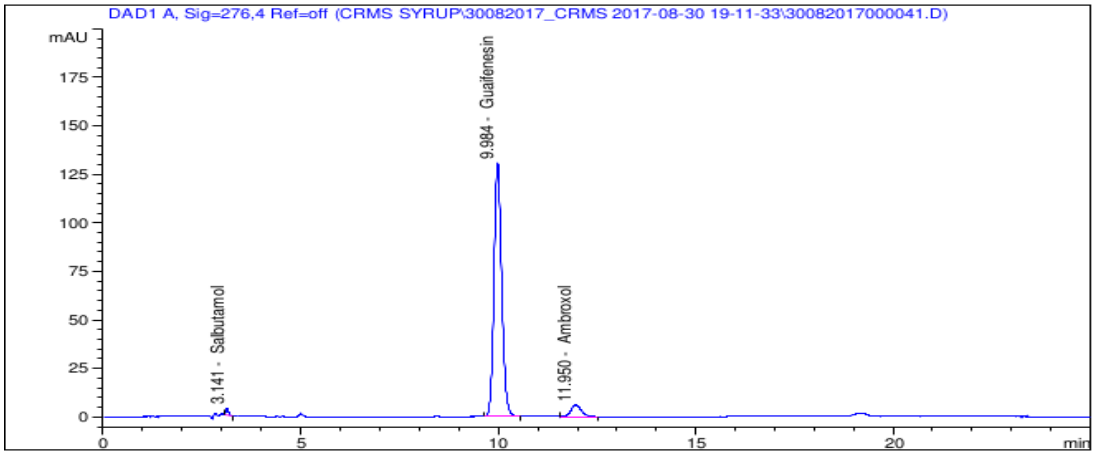
Level 50%-3_1



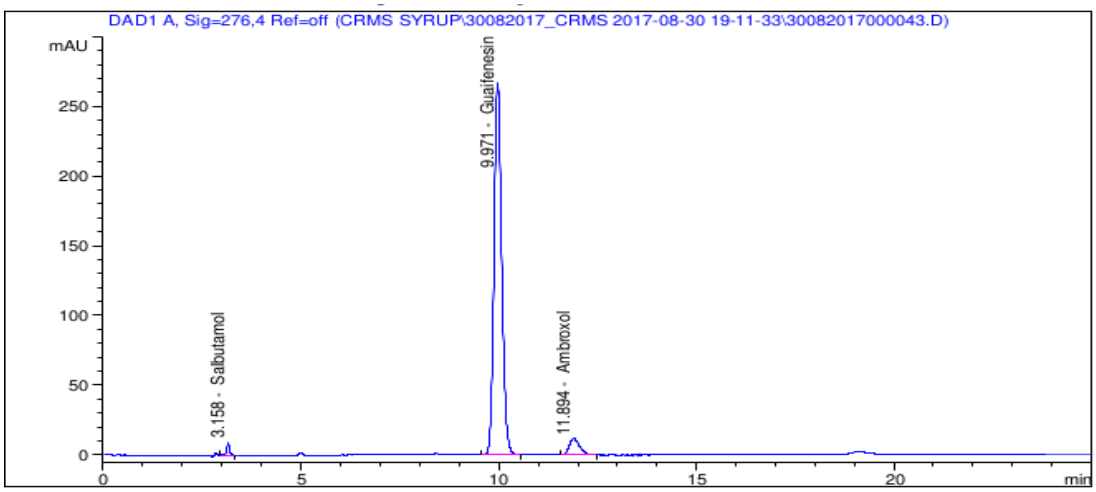
Level 50%-3_2



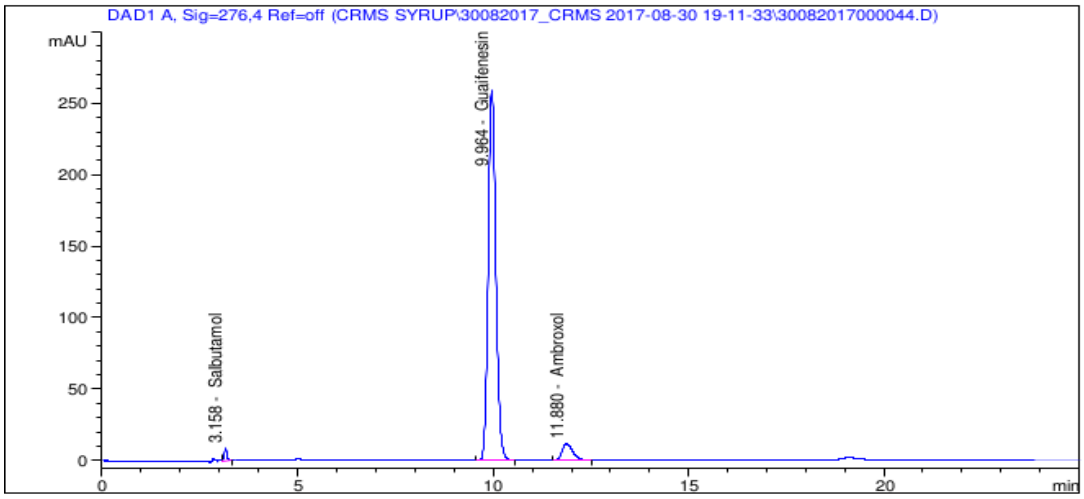
Level 100%-1_1



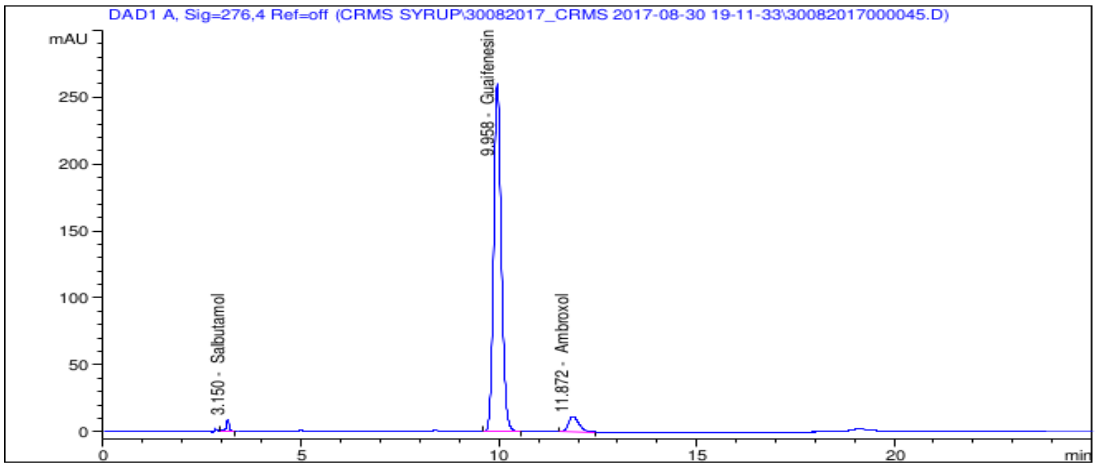
Level 100%-1_2



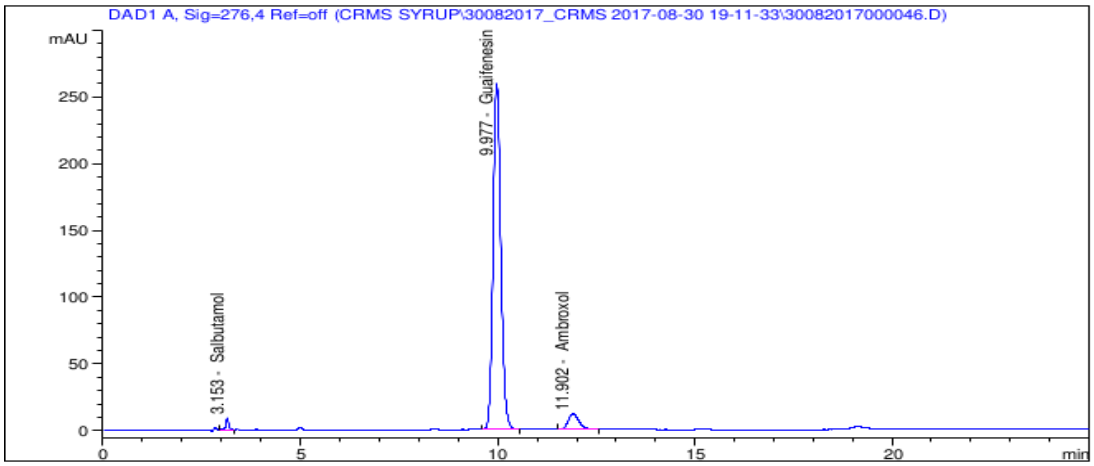
Level 100%-2_1



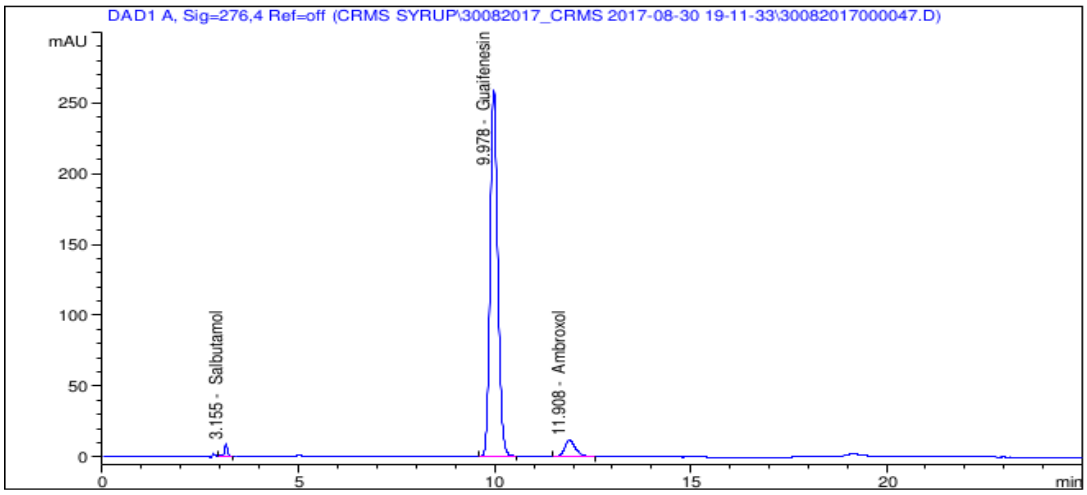
Level 100%-2_2



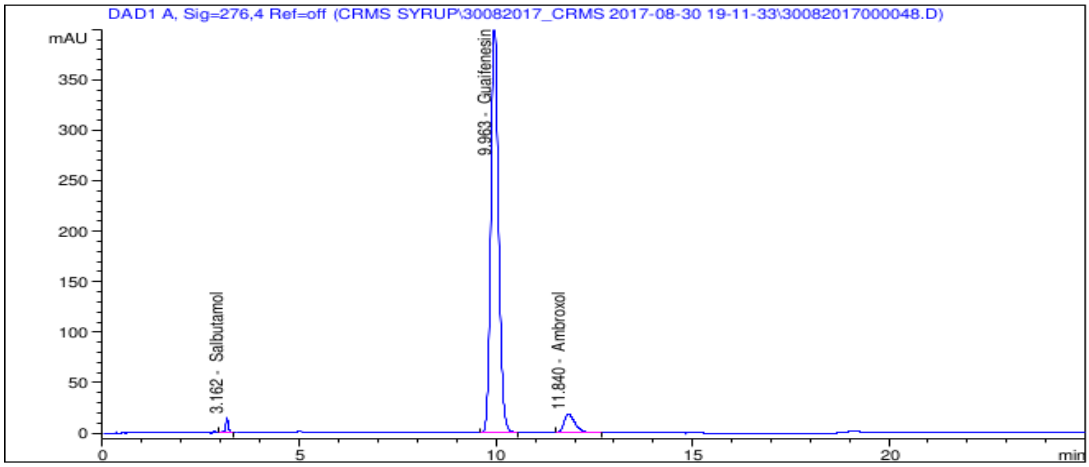
Level 100%-3_1



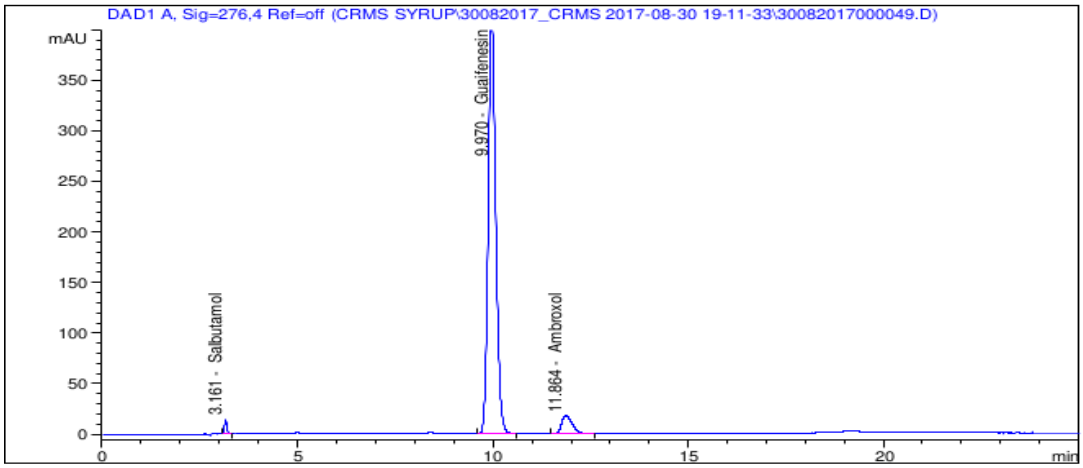
Level 100%-3_2



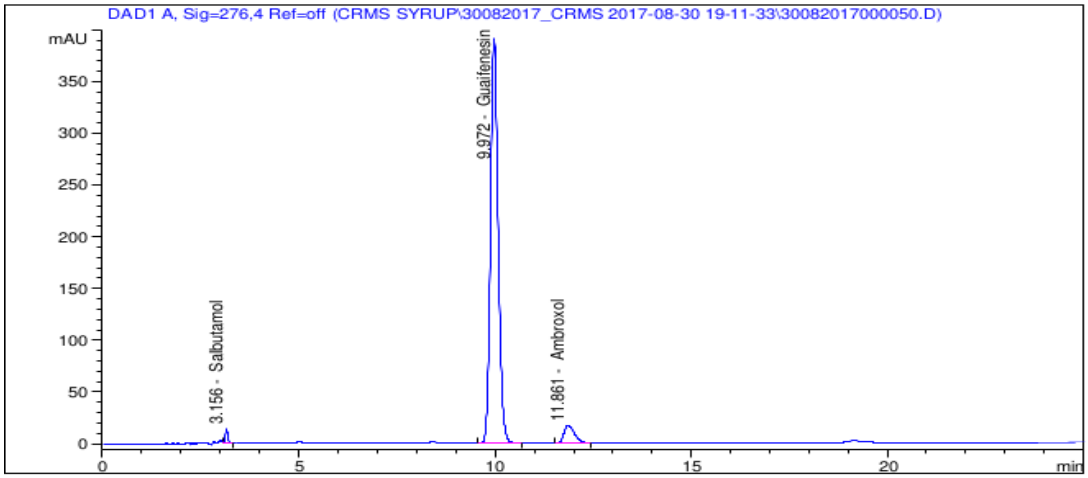
Level 150%-1_1



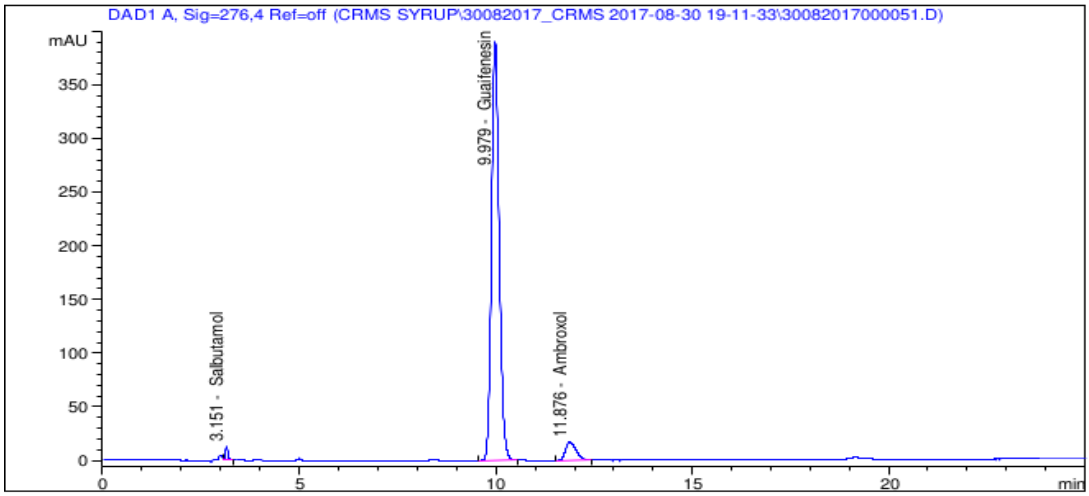
Level 150%-1_2



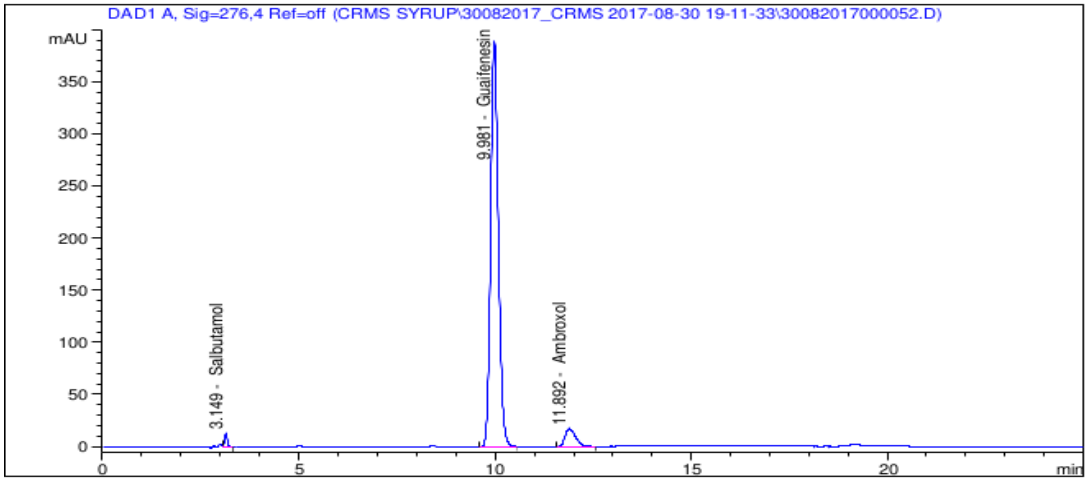
Level 150%-2_1



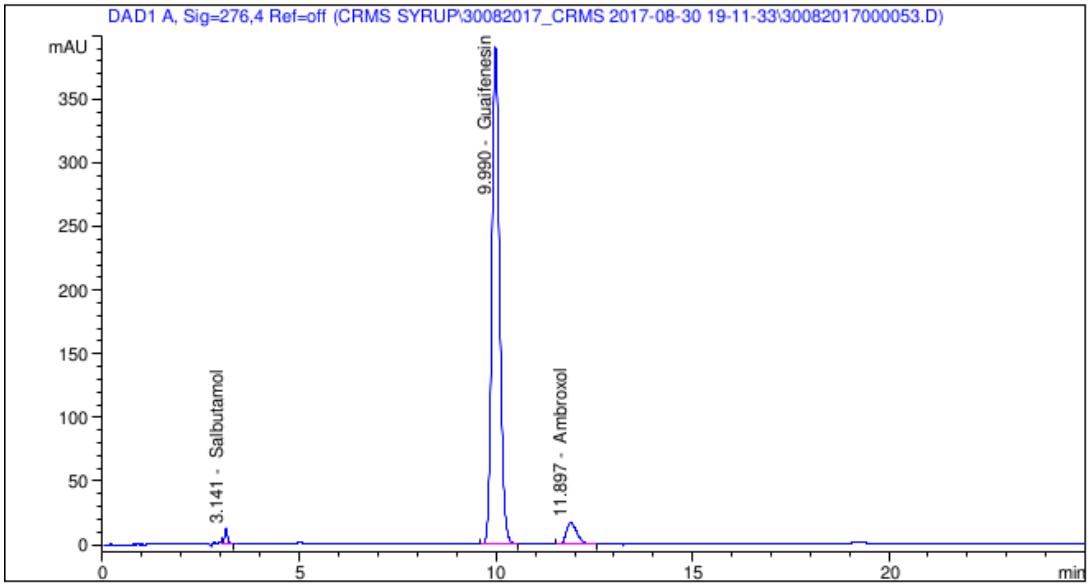
Level 150%-2_2



Level 150%-3_1



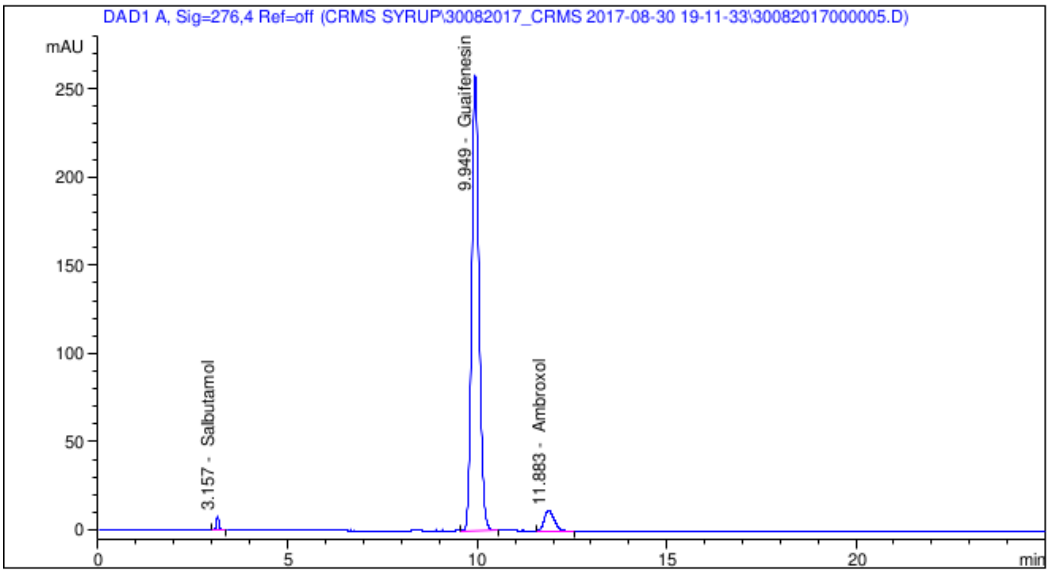
Level 150%-3_2



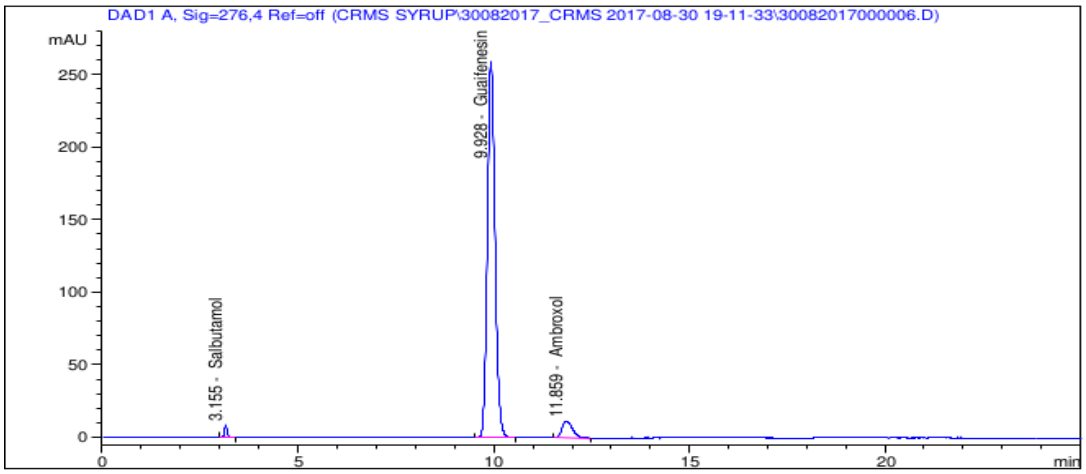
Precision

1. System Precision

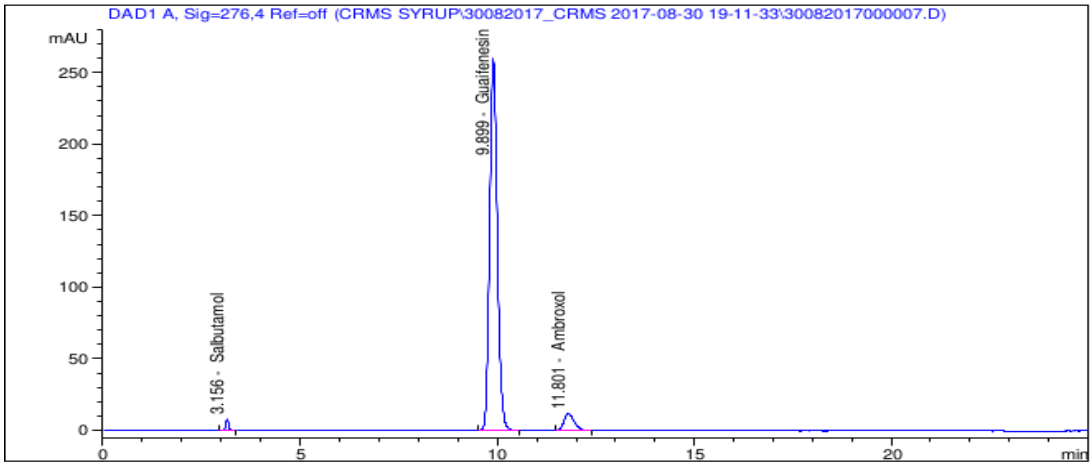
Standard_1



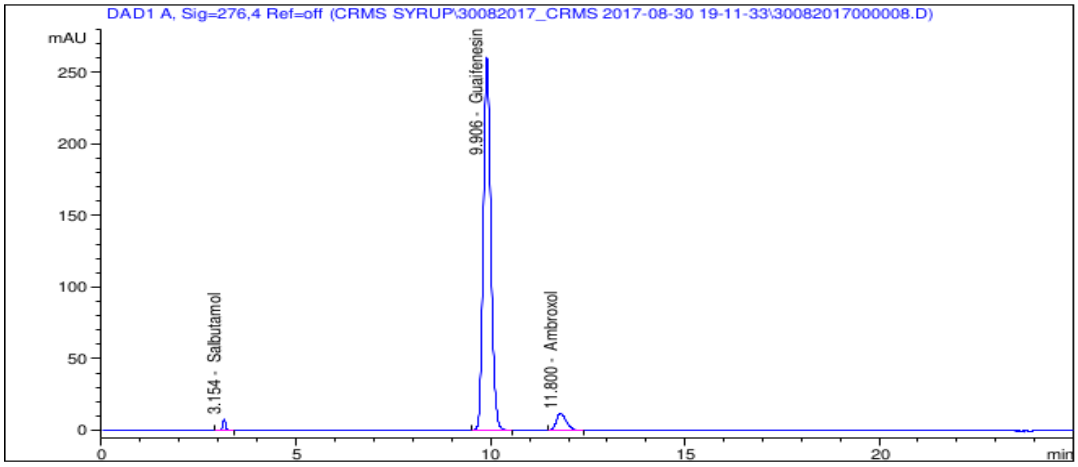
Standard_2



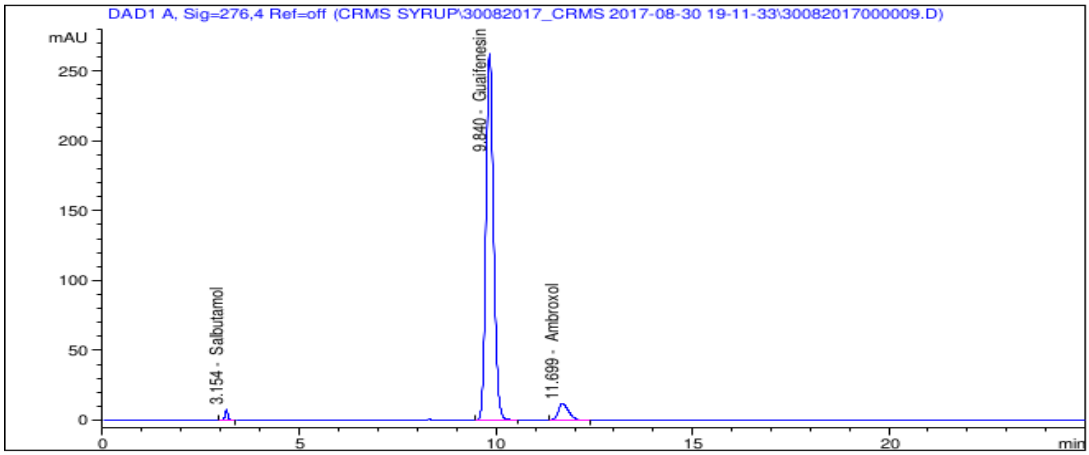
Standard_3



Standard_4

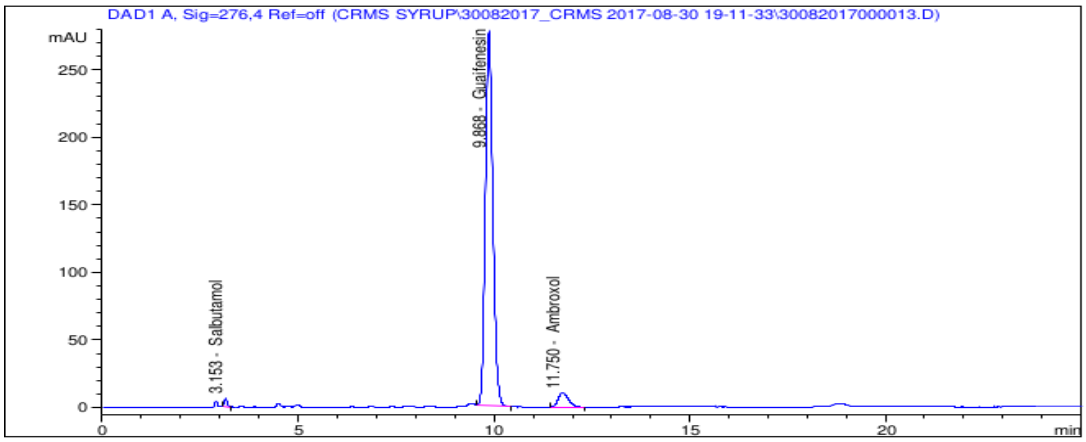


Standard_5

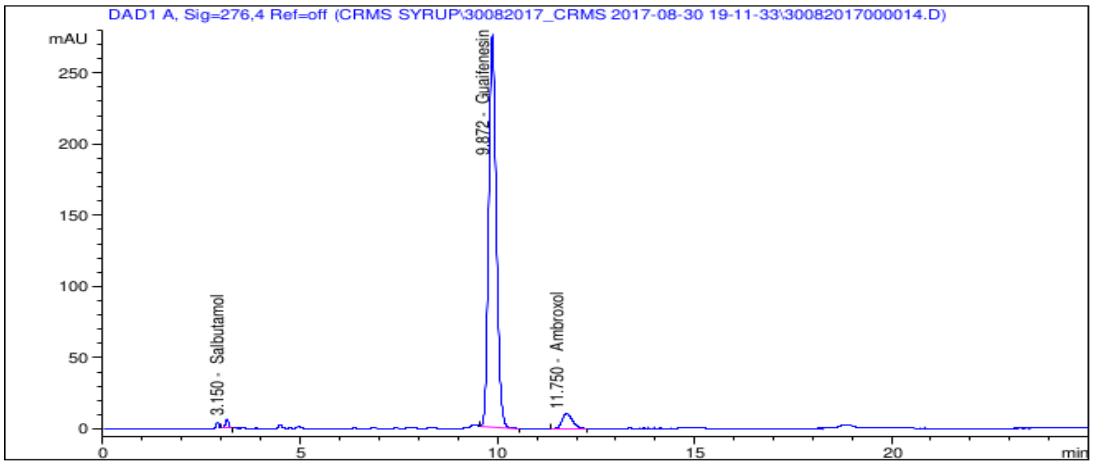


2. Method precision

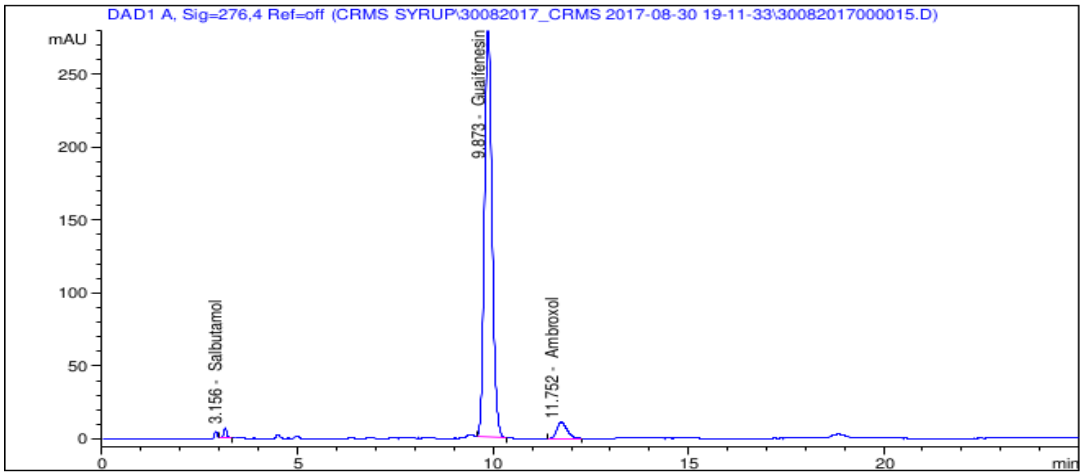
Method precision-1_1



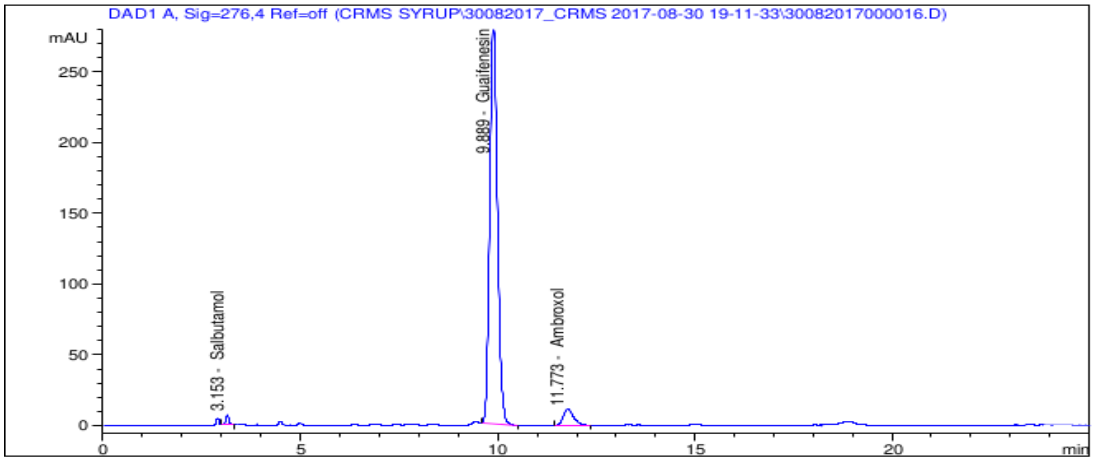
Method precision-1_2



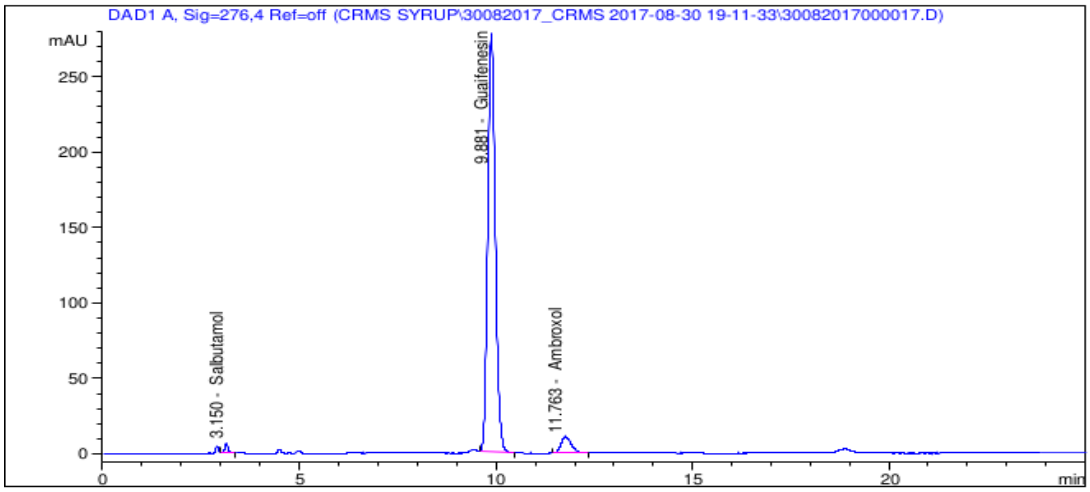
Method precision -2_1



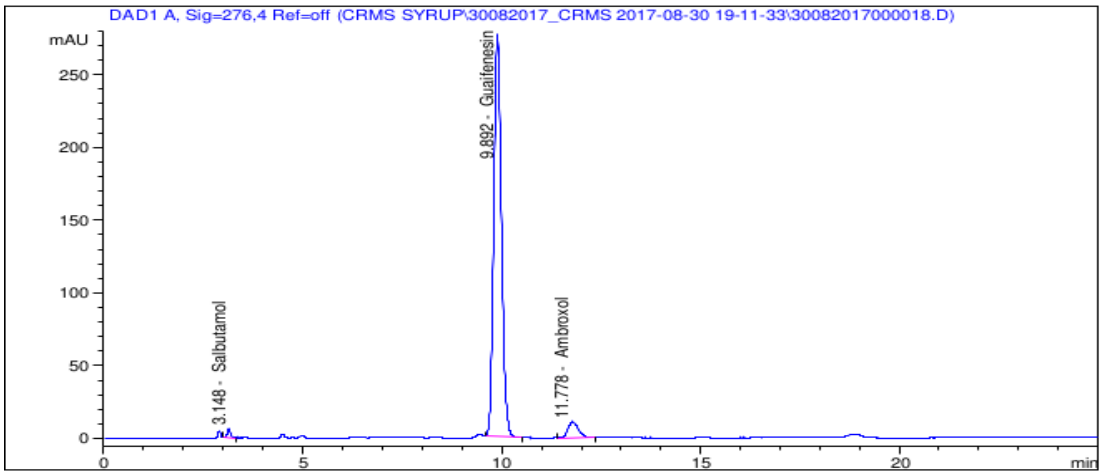
Method precision-2_2



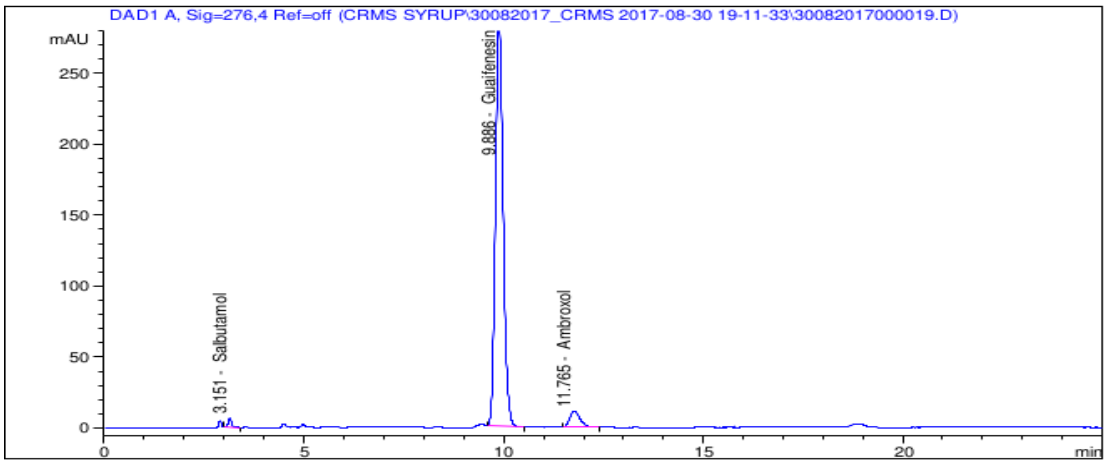
Method precision-3_1



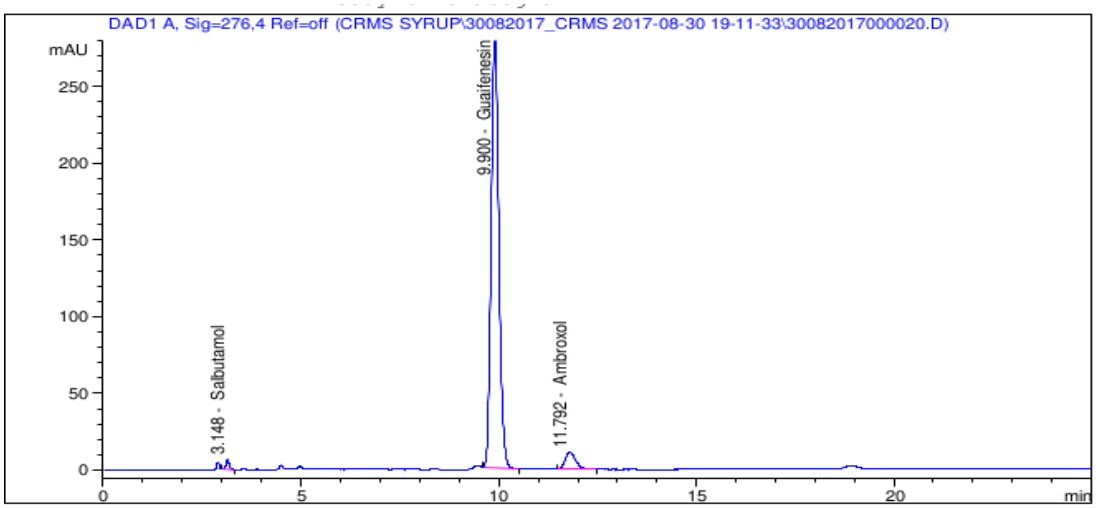
Method precision-3_2



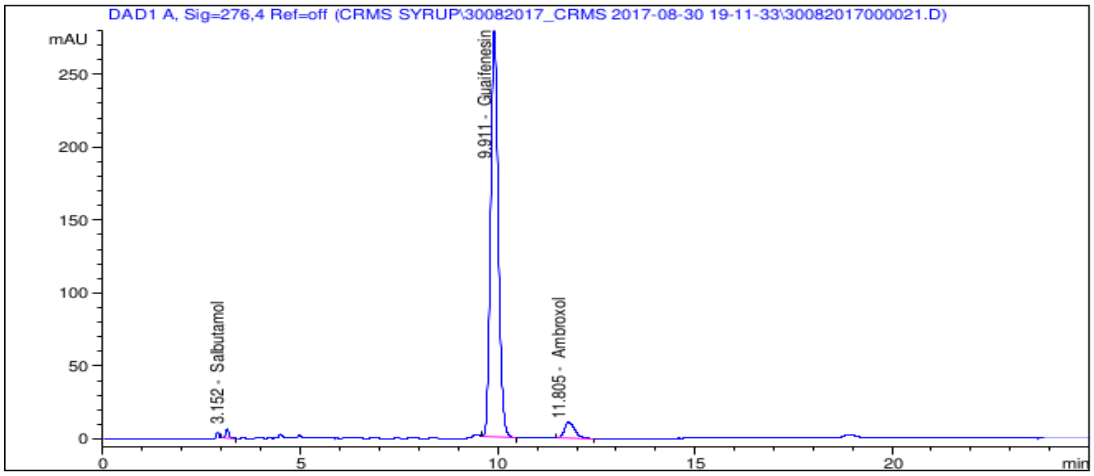
Method precision-4_1



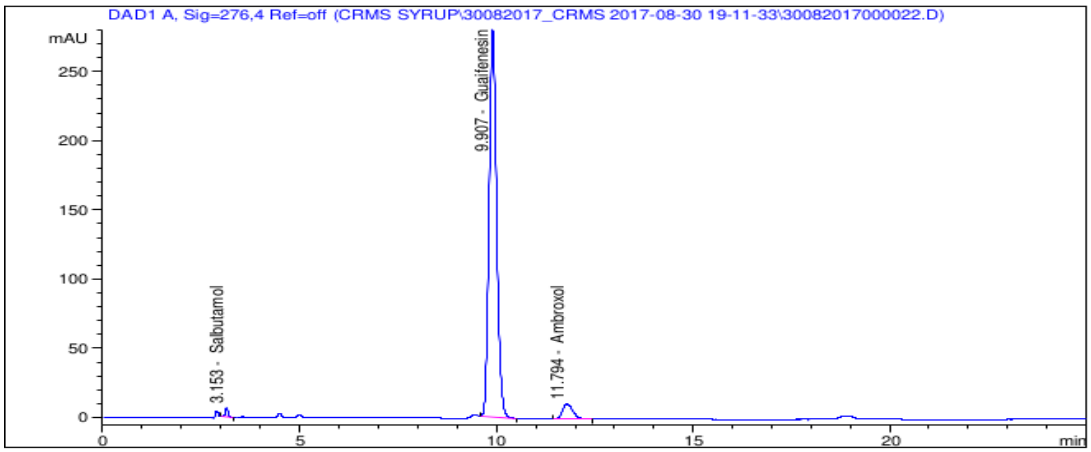
Method precision-4_2



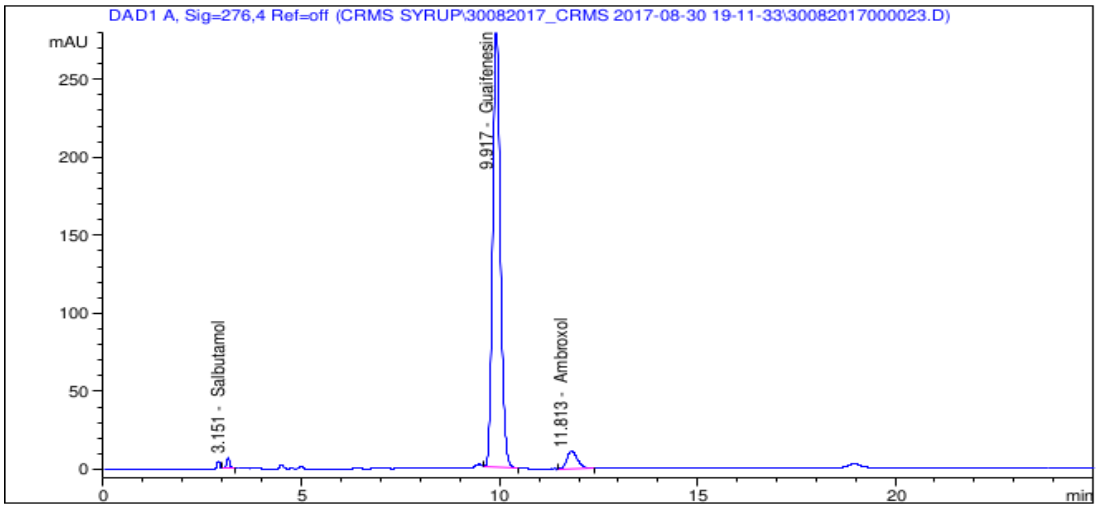
Method precision-5_1



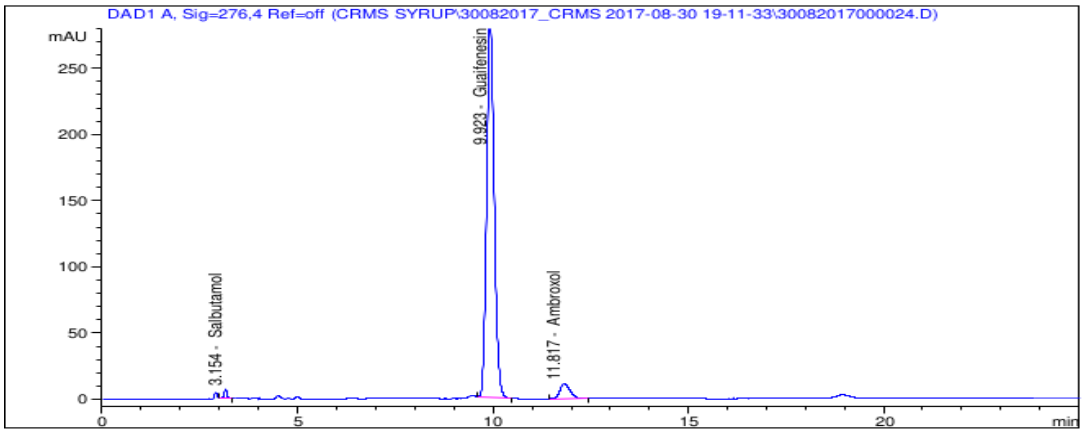
Method precision-5_2



Method precision-6_1

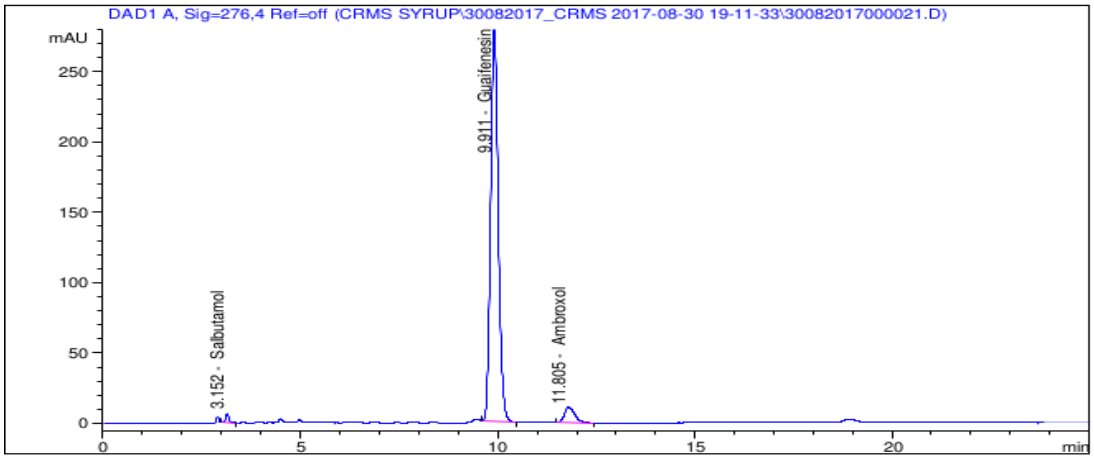


Method precision-6_2

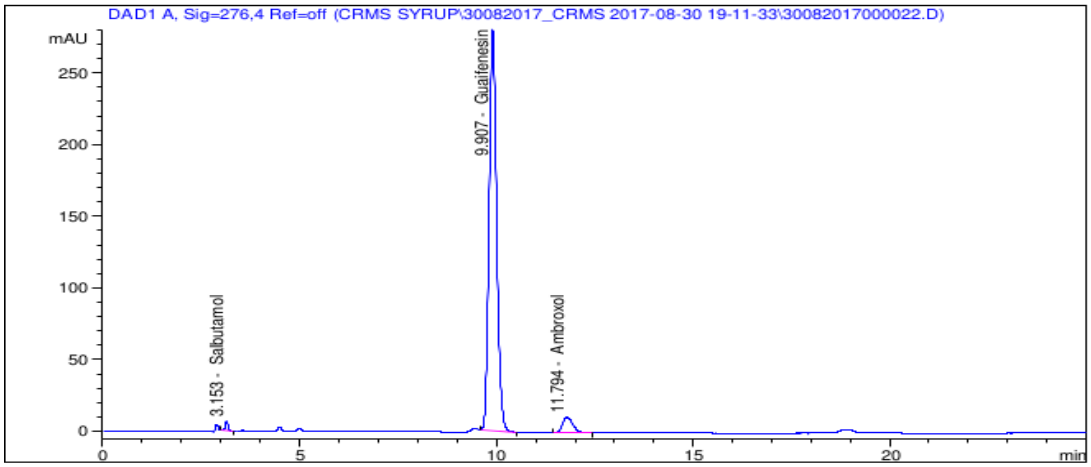


Intermediate Precision

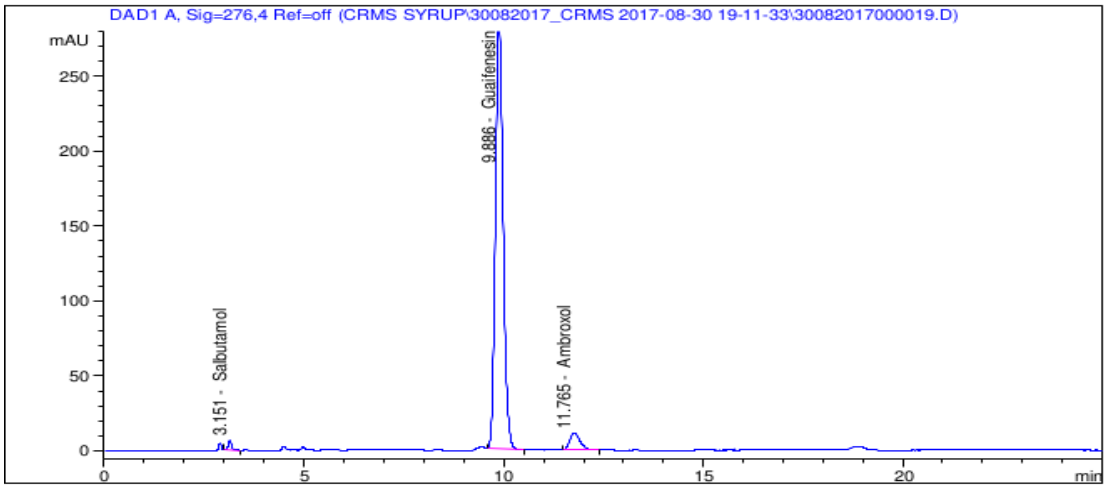
Intermediate precision-1_1



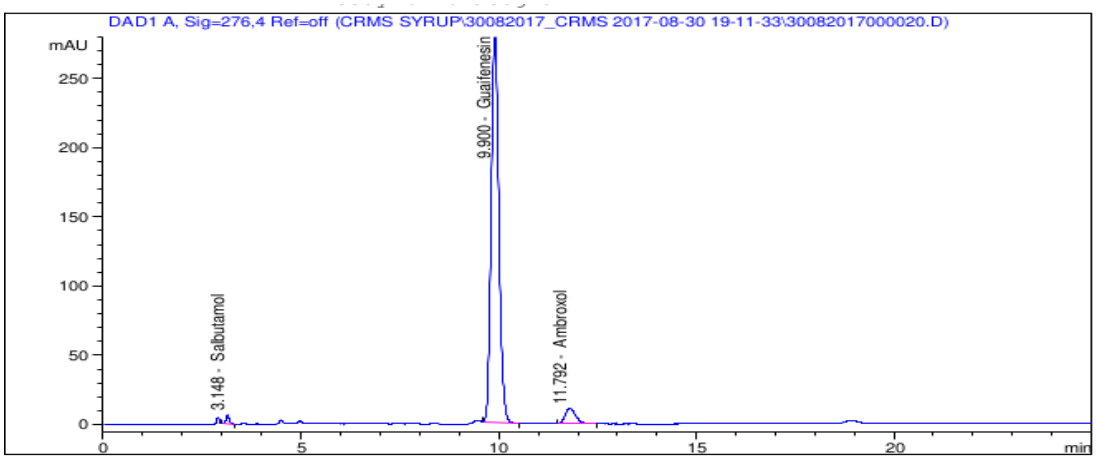
Intermediate precision-1_2



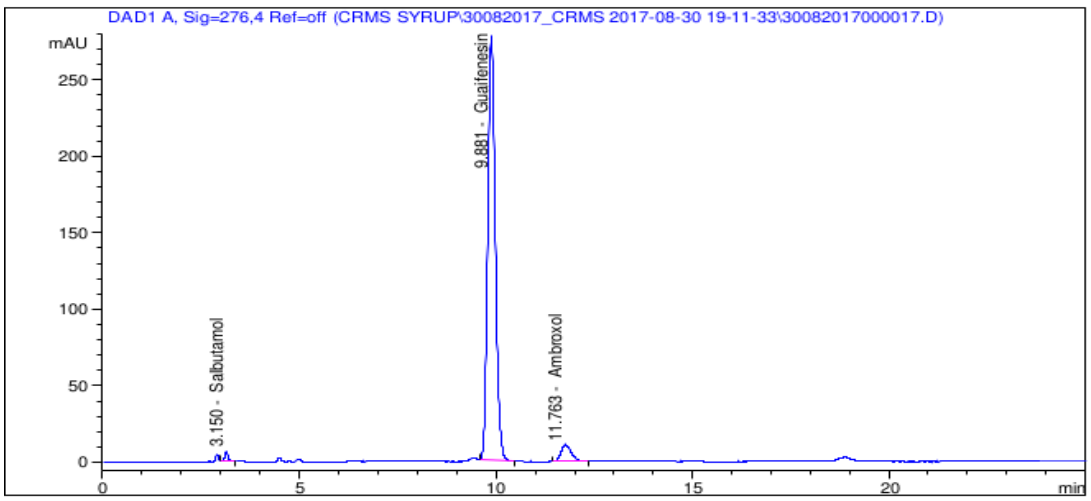
Intermediate precision-2_1



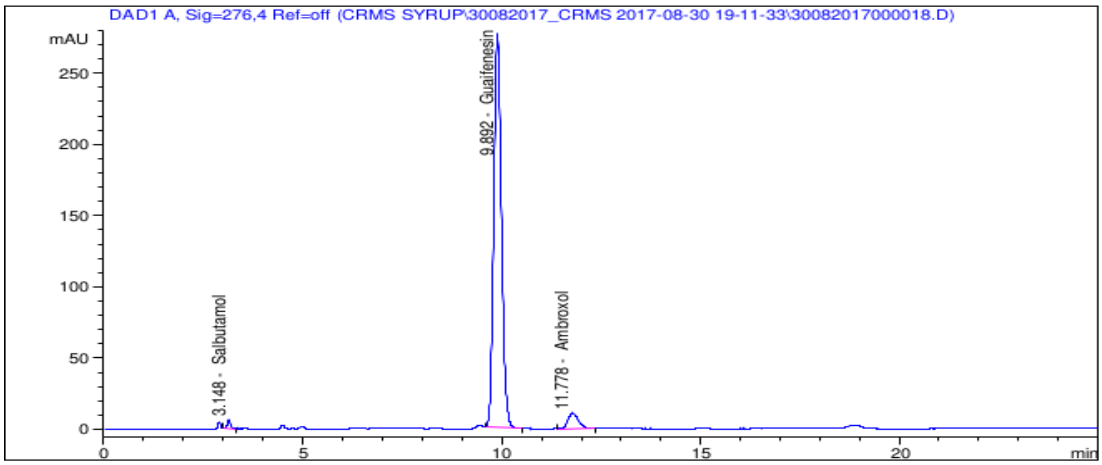
Intermediate precision-2_2



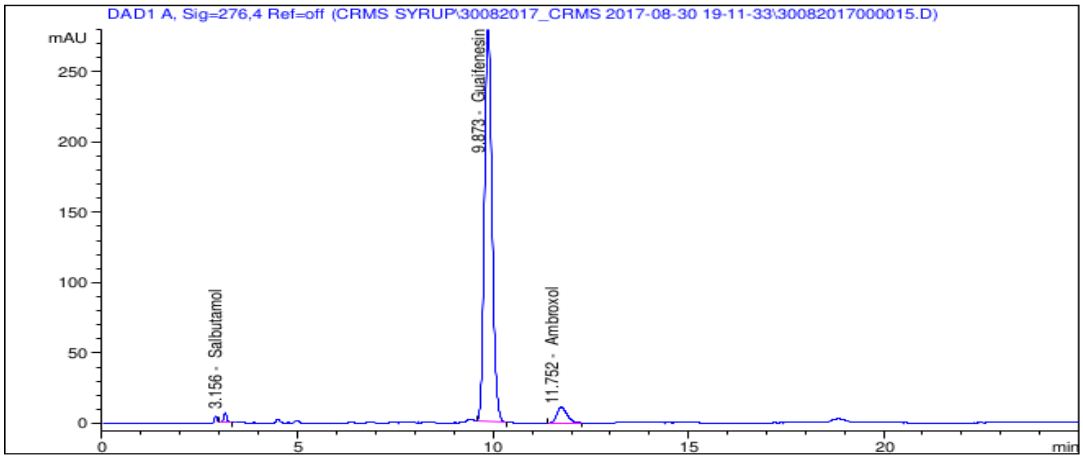
Intermediate precision-3_1



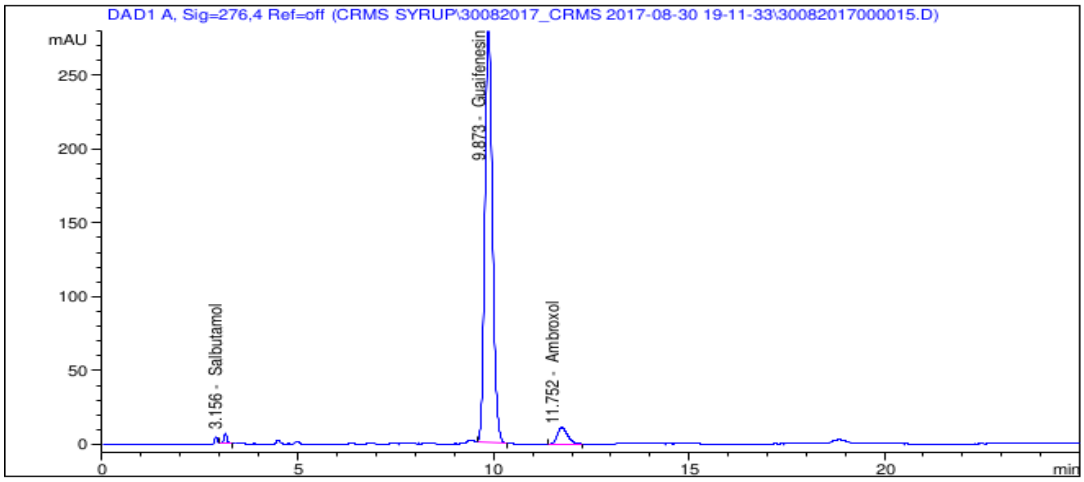
Intermediate precision-3_2



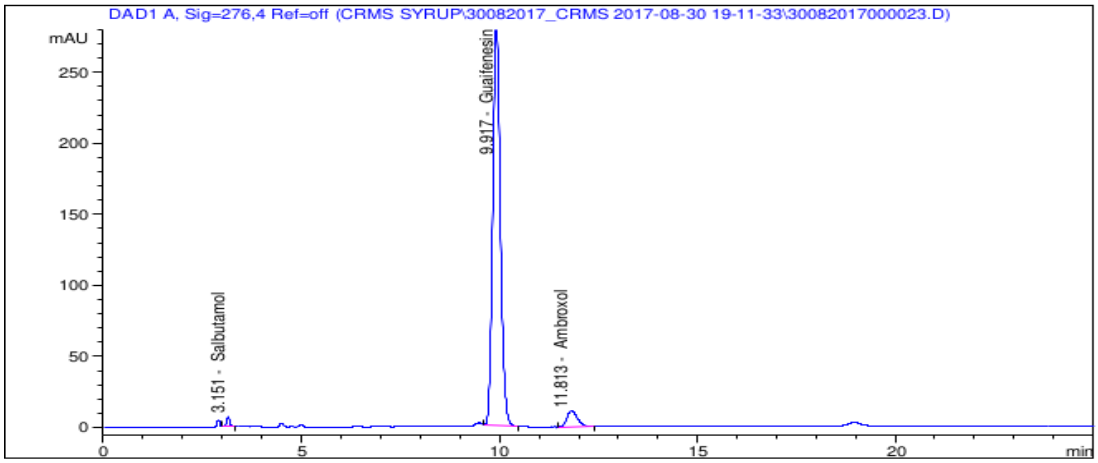
Intermediate precision-4_1



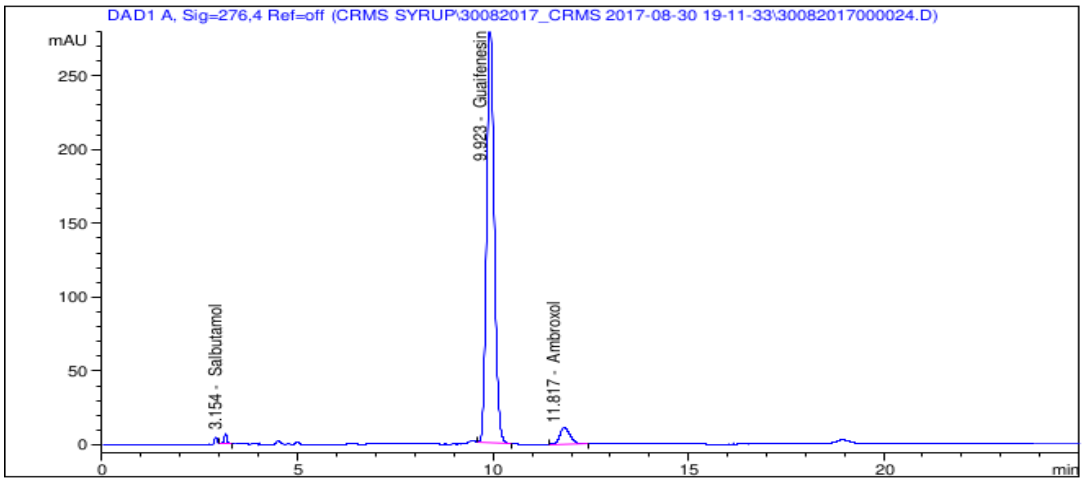
Intermediate precision-4_2



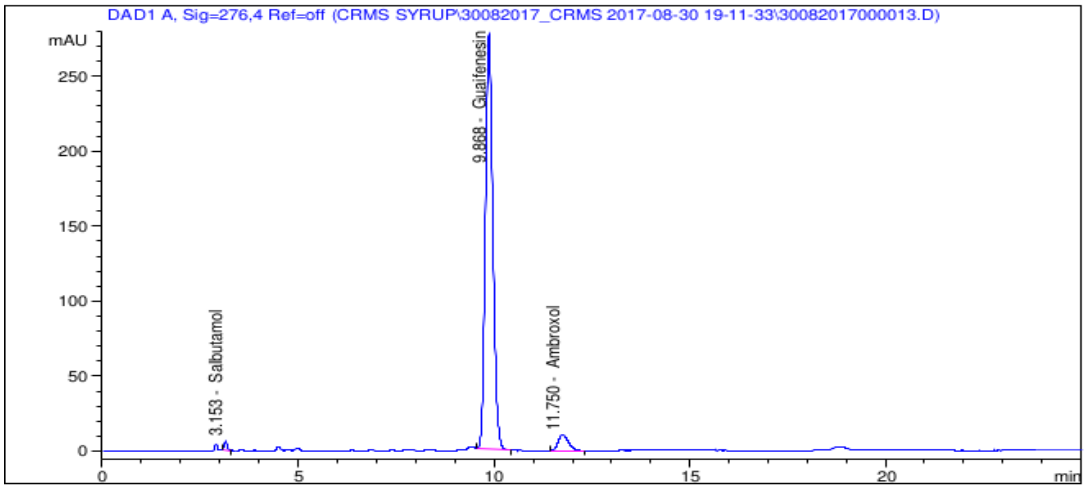
Intermediate precision-5_1



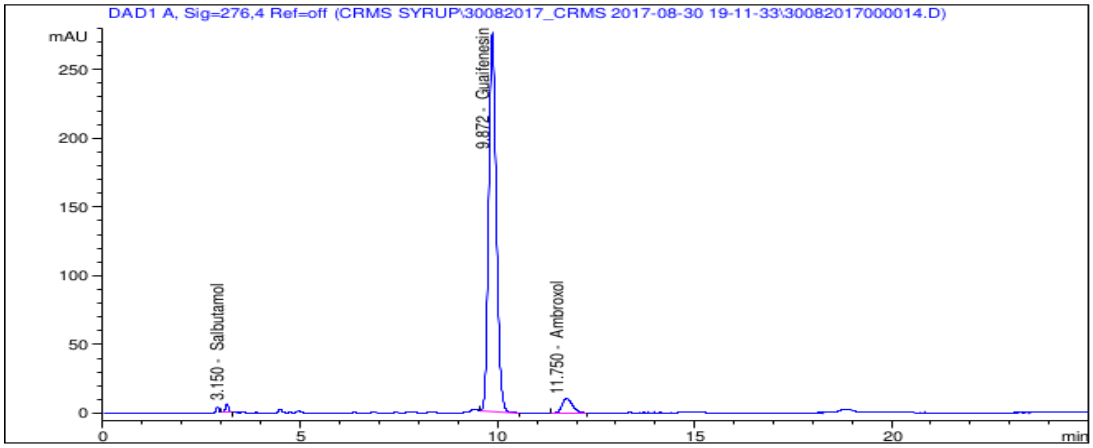
Intermediate precision-5_2



Intermediate precision-6_1

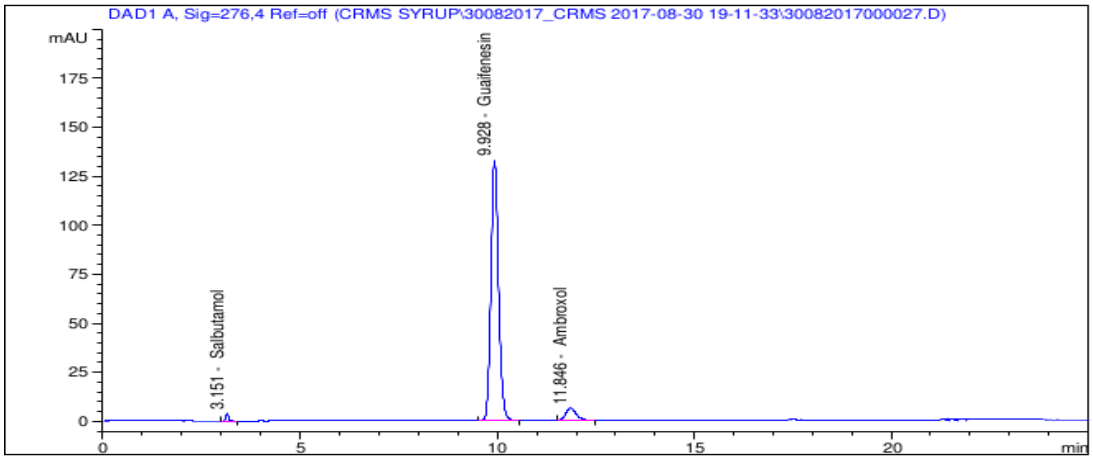


Intermediate precision-6_2

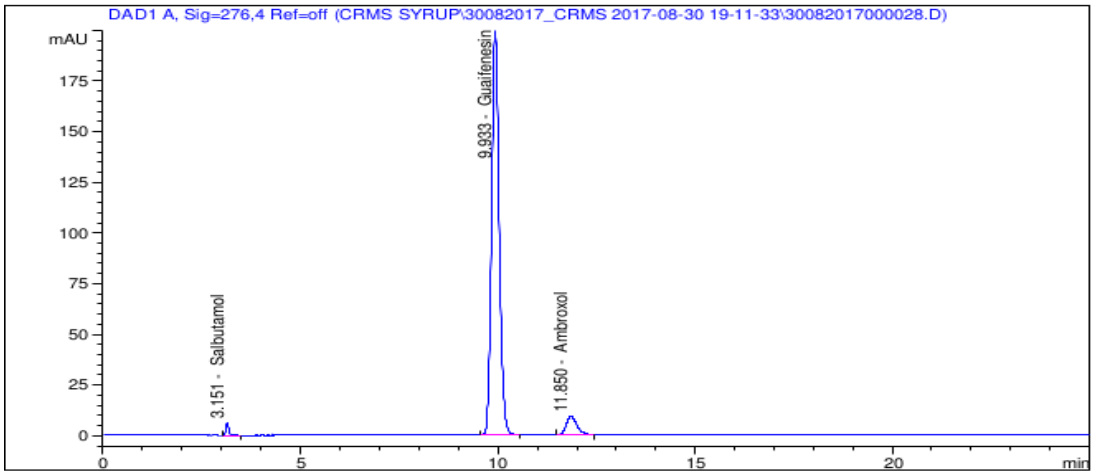


Linearity

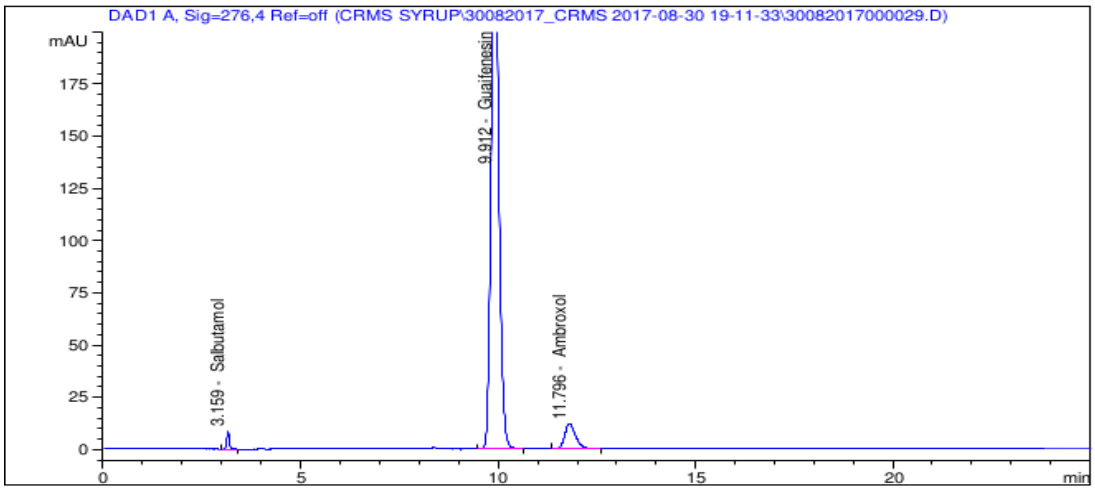
Level 1-50%



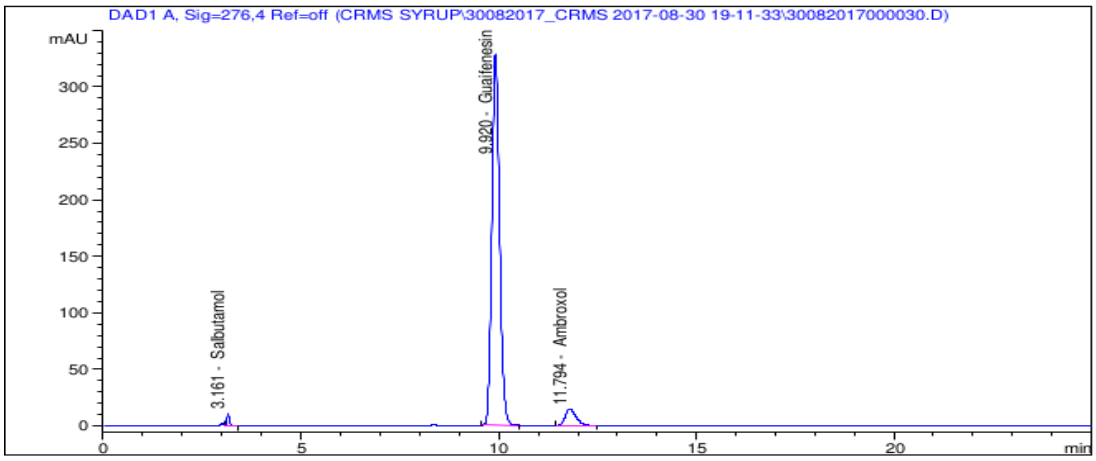
Level 2-75%



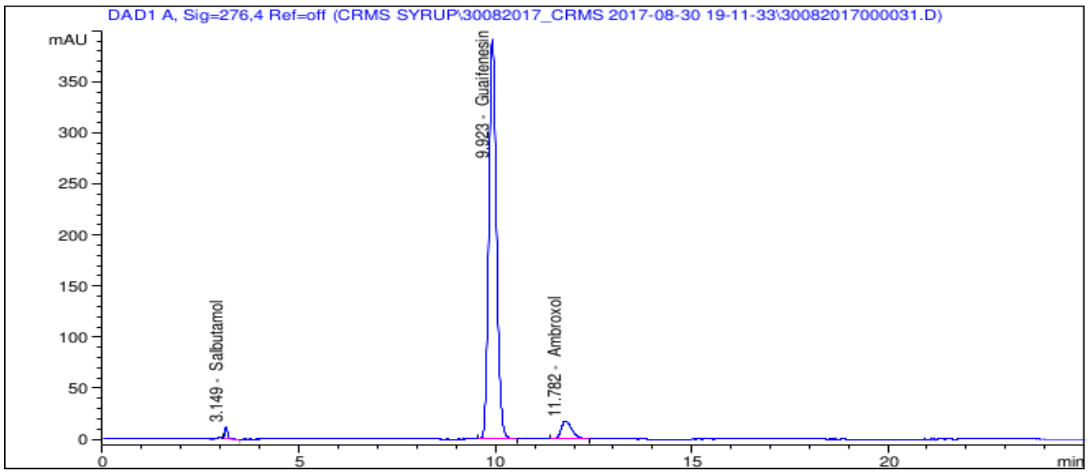
Level 3-100%



Level 4-125%

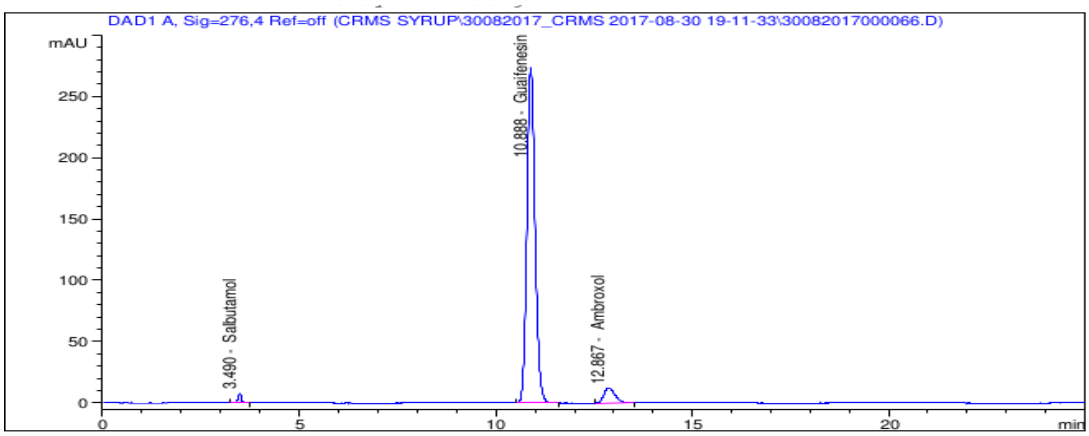


Level 5-150%

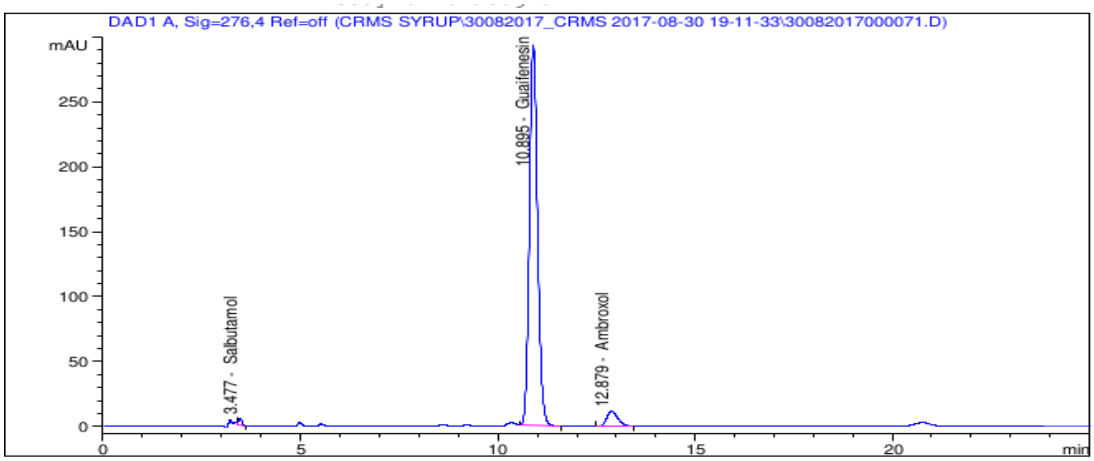


Robustness

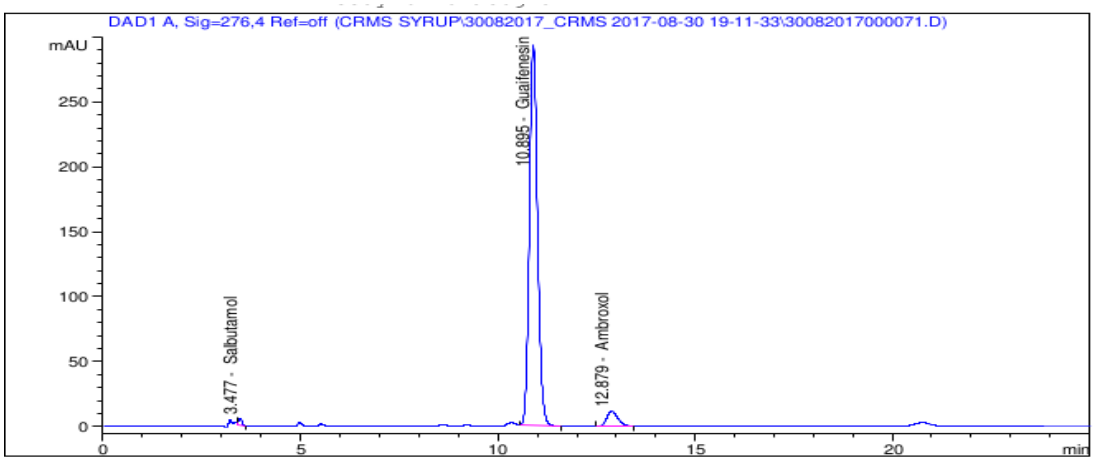
Flow minus_Standard (0.9mL/Min)



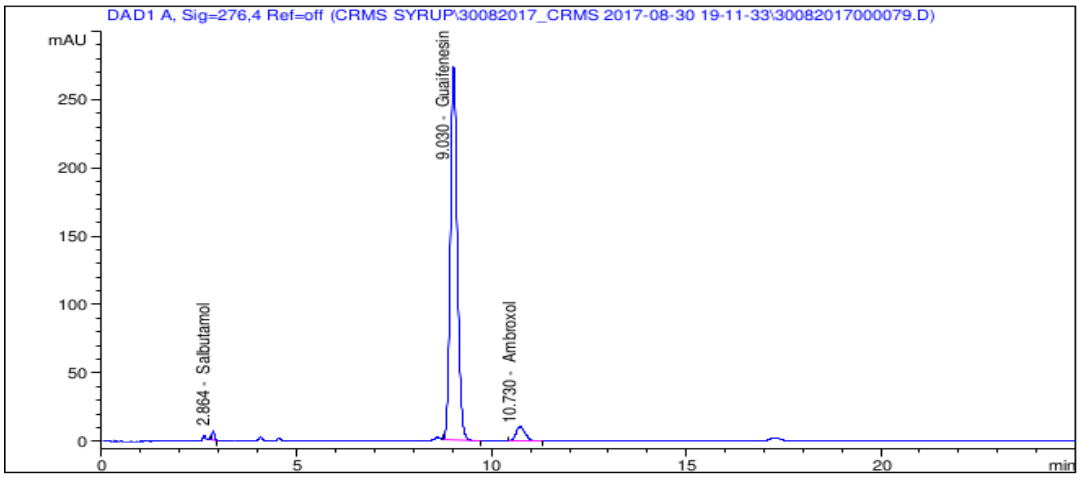
Flow minus_Sample (0.9mL/Min)



Flow plus_Standard (1.1mL/Min)

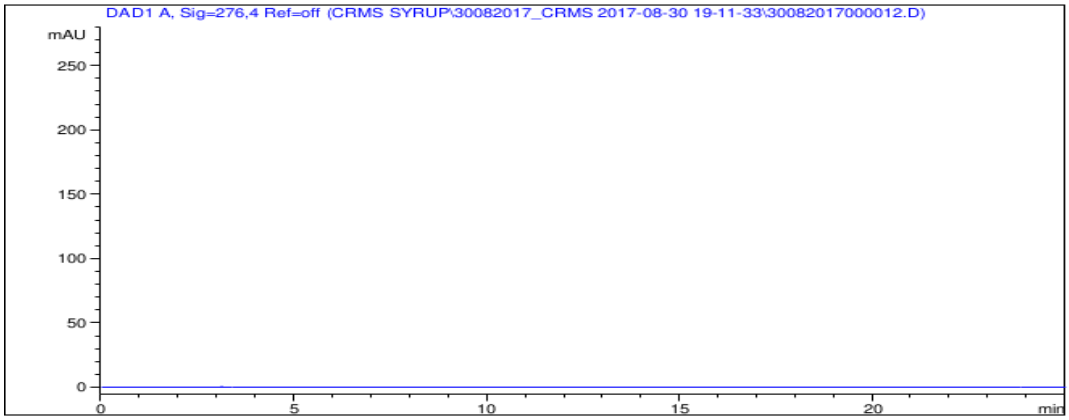


Flow plus_Sample (1.1mL/Min)

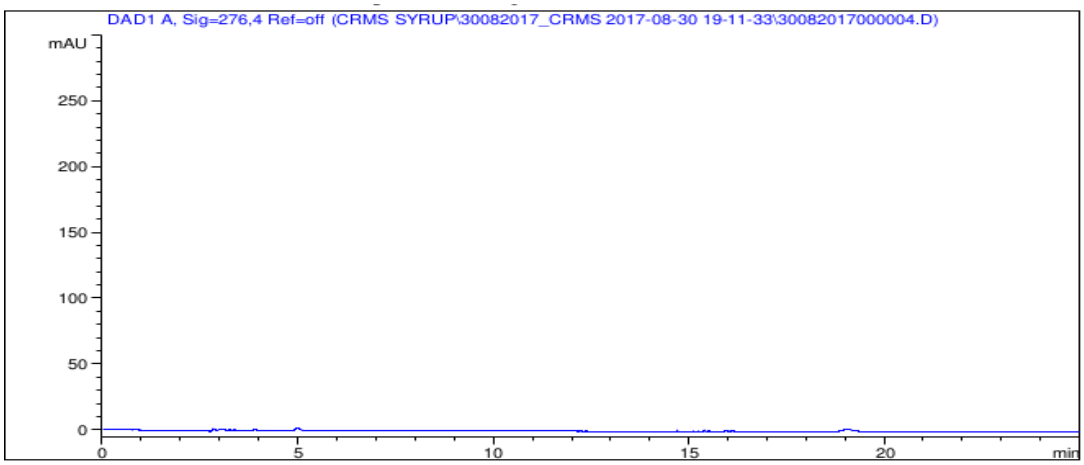


Specificity

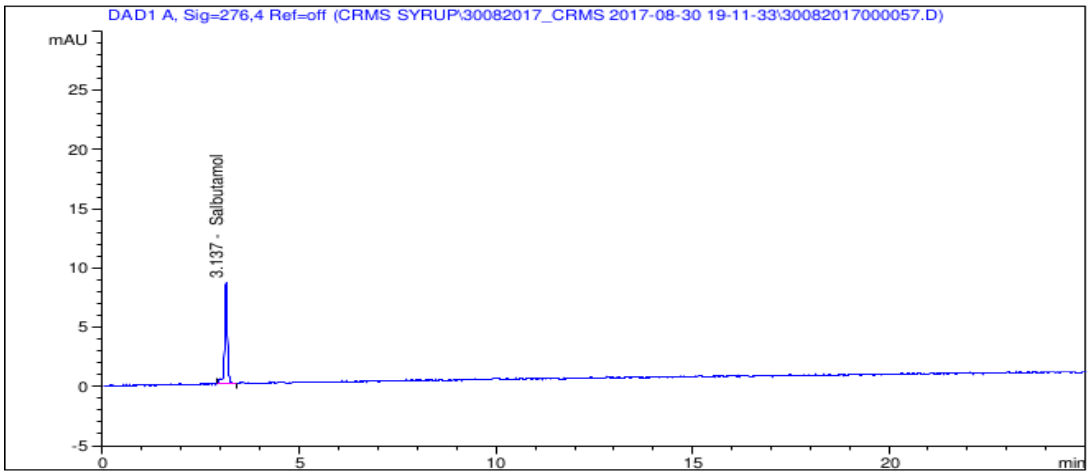
Blank



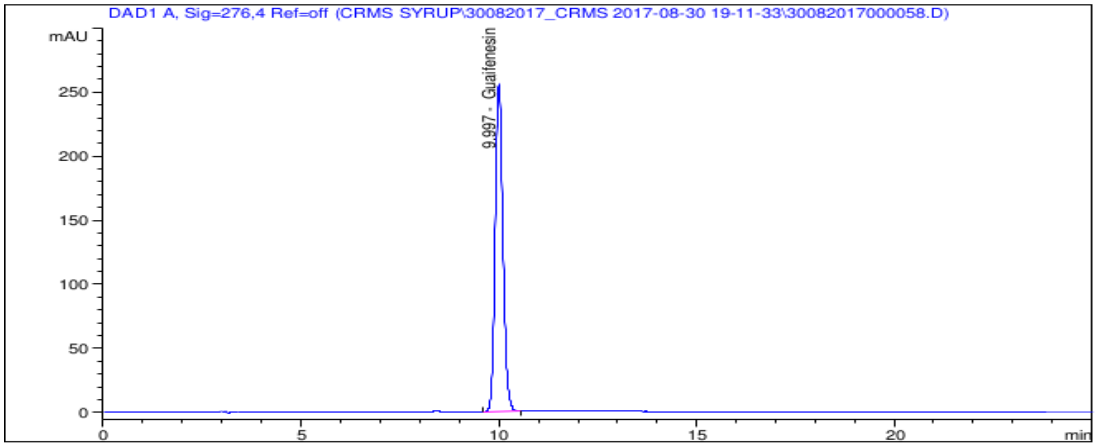
Placebo



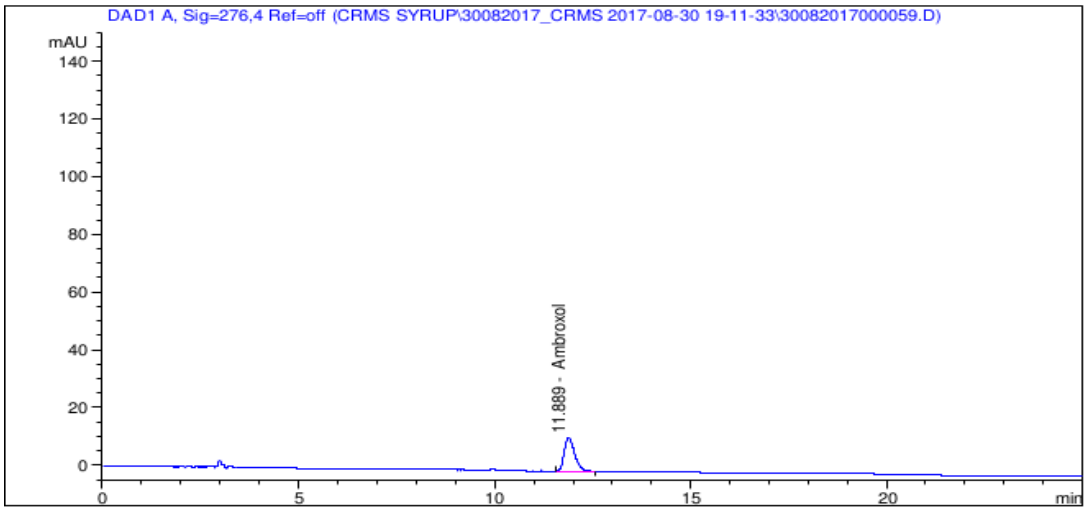
Standard_Salbutamol sulphate



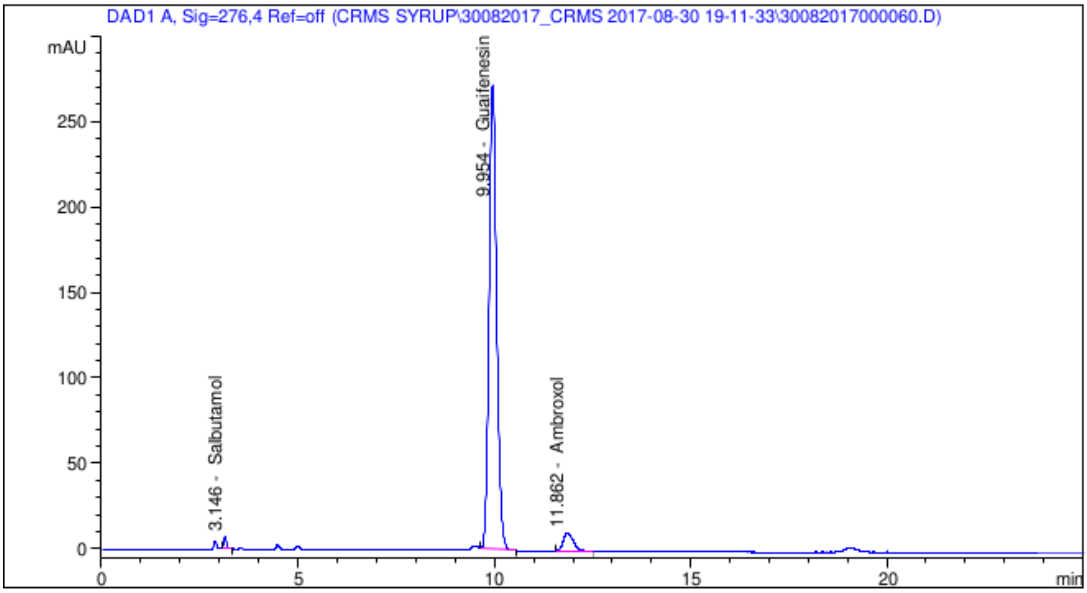
Standard_Guaifenesin



Standard_Ambroxol hydrochloride

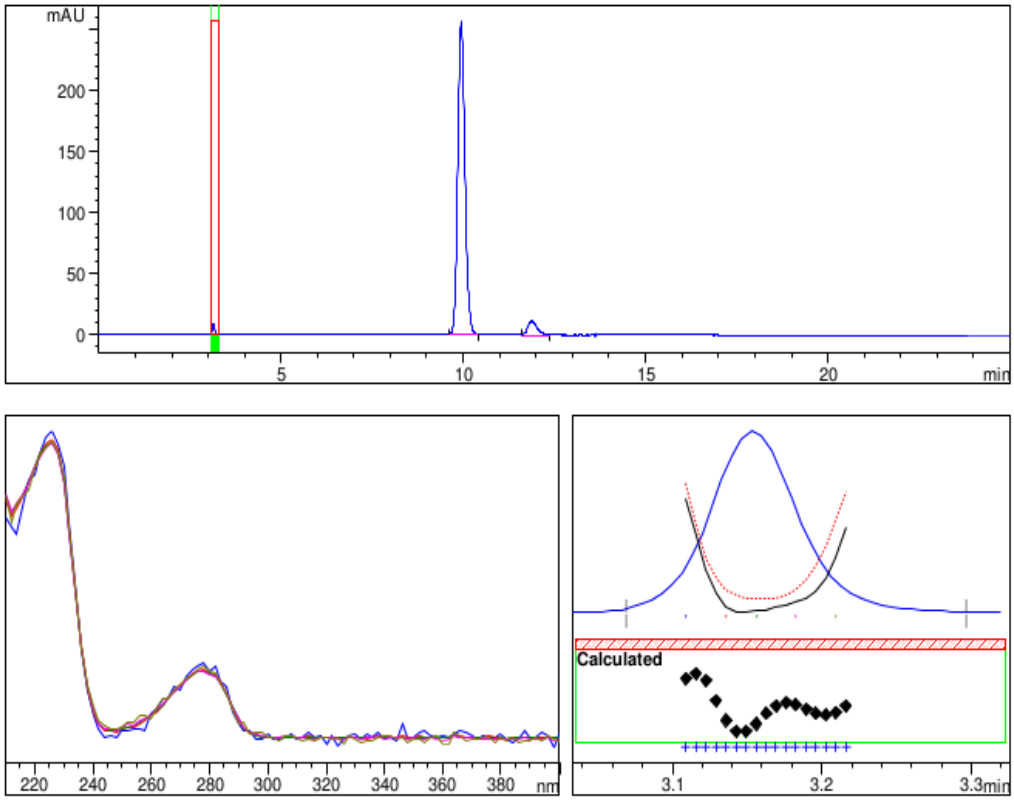


Control Sample

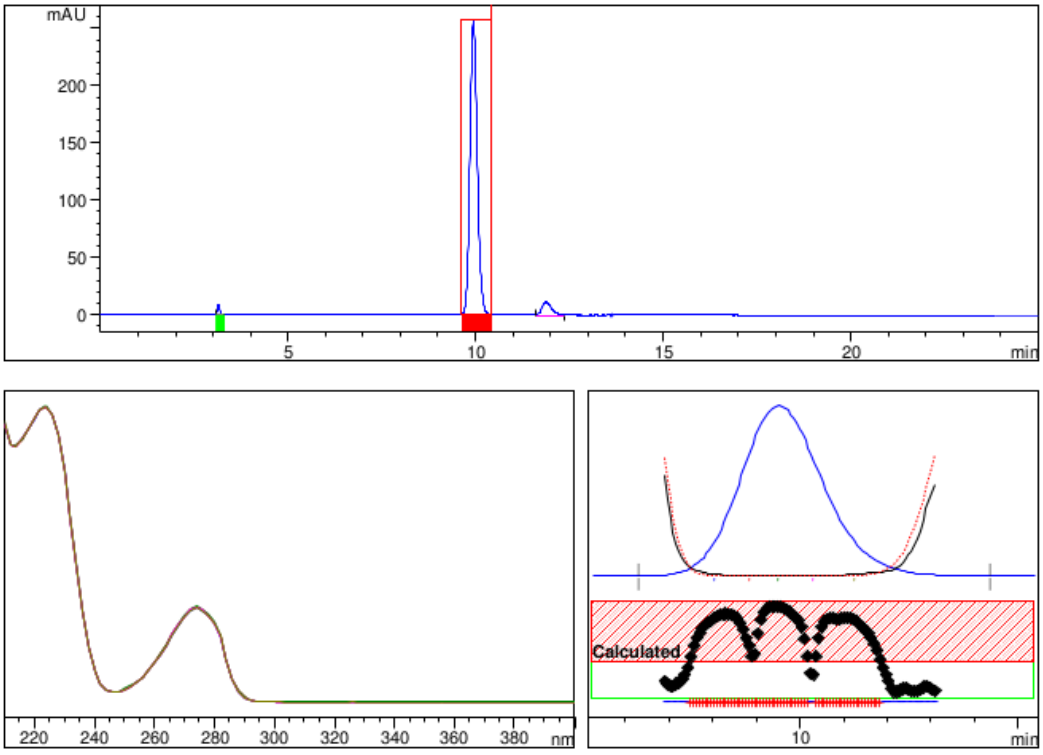


Peak Purity

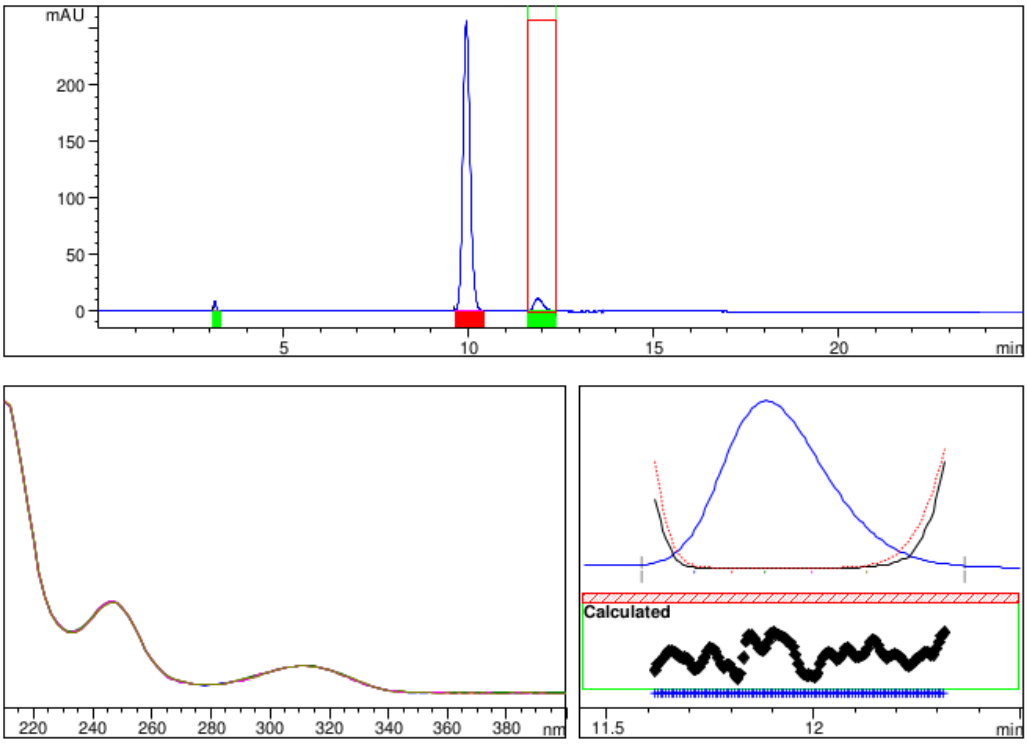
Salbutamol sulphate



Guaifenesin

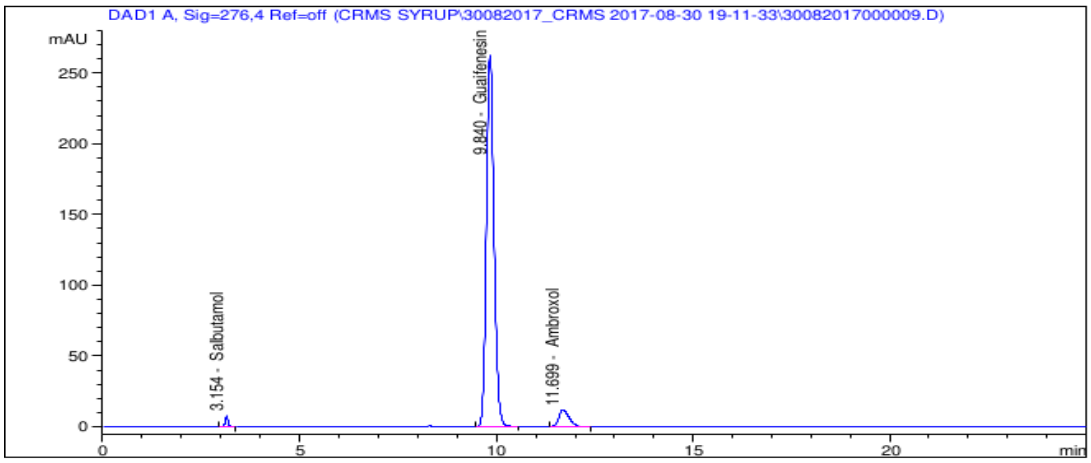


Ambroxol hydrochloride

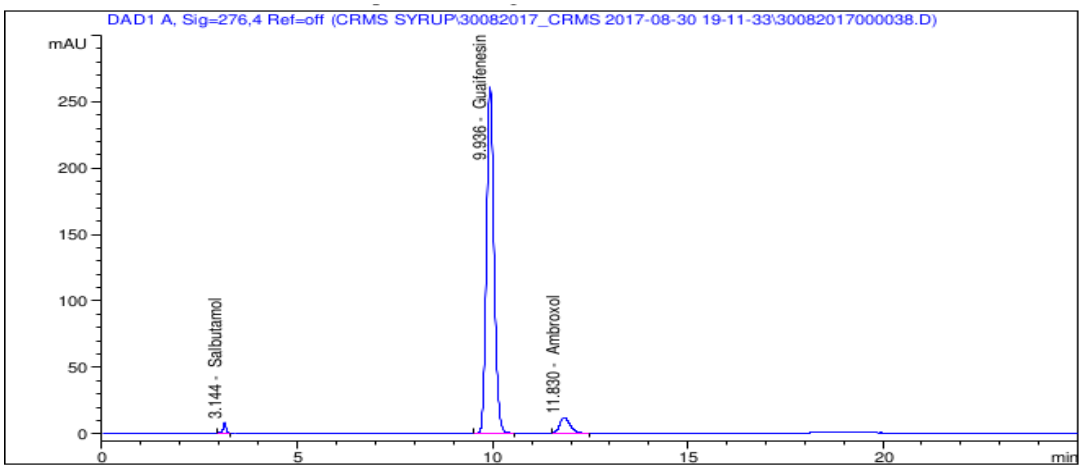


Solution stability

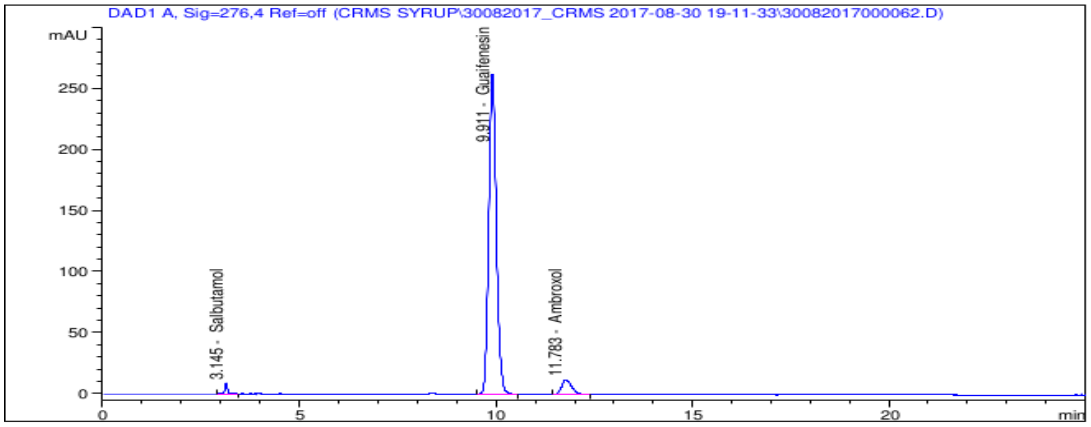
Standard_Initial



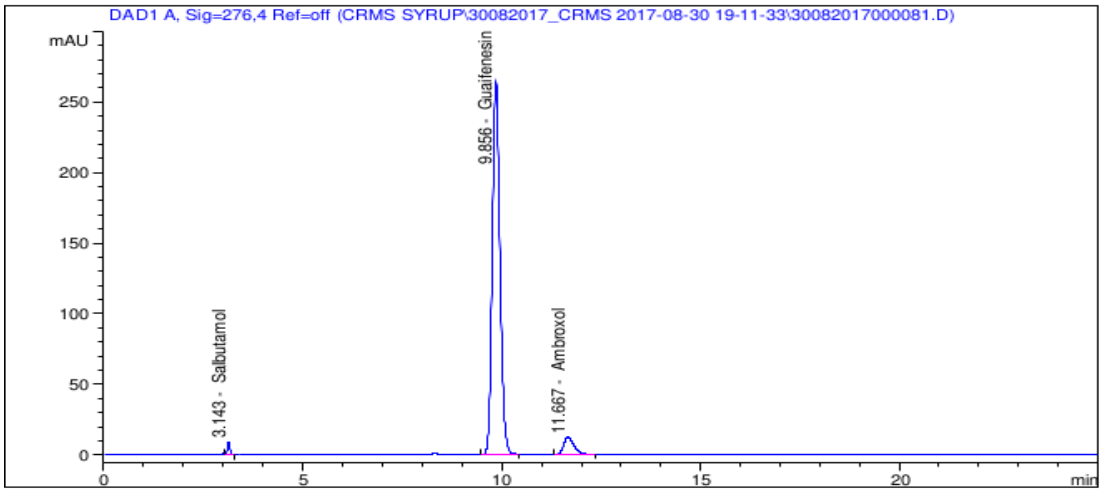
Standard_24Hrs



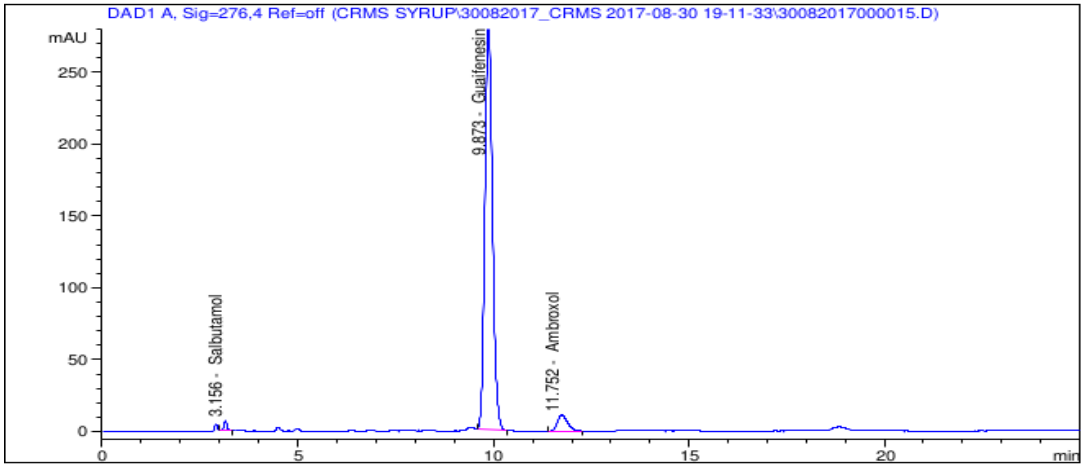
Standard_36Hrs



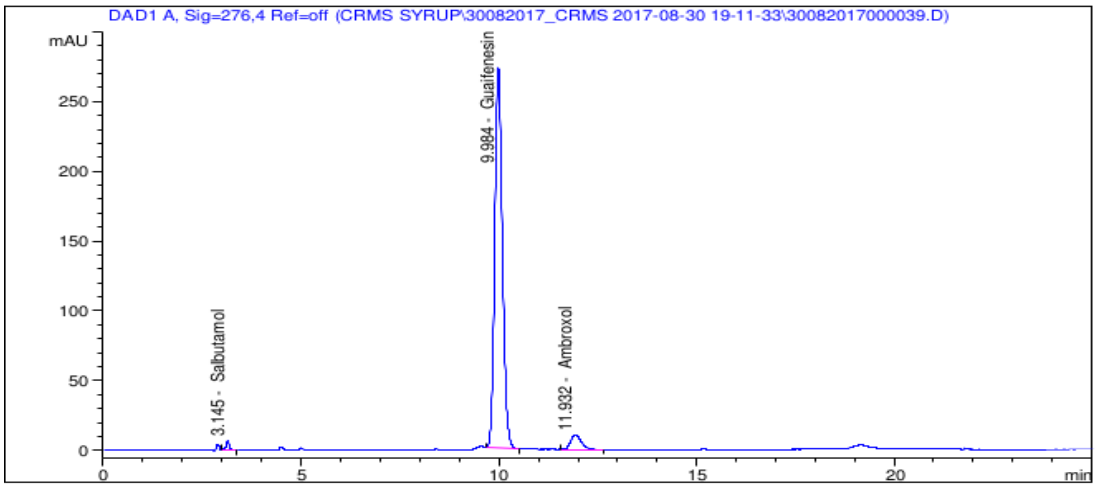
Standard_48Hrs



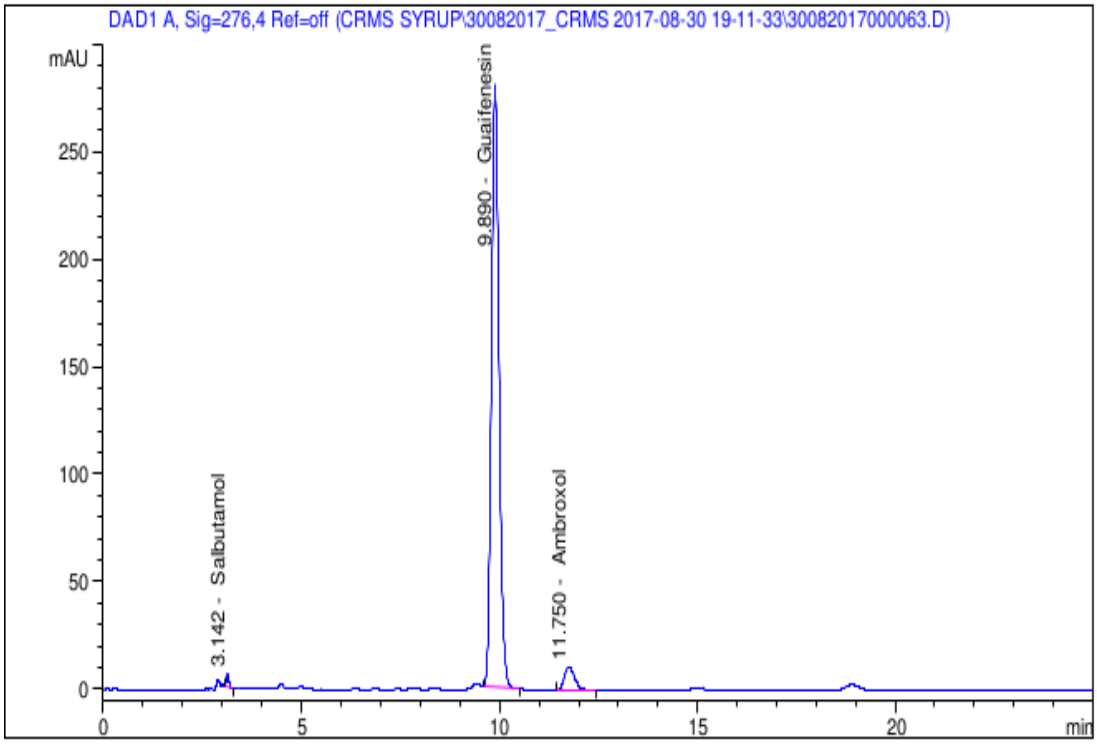
Sample_Initial



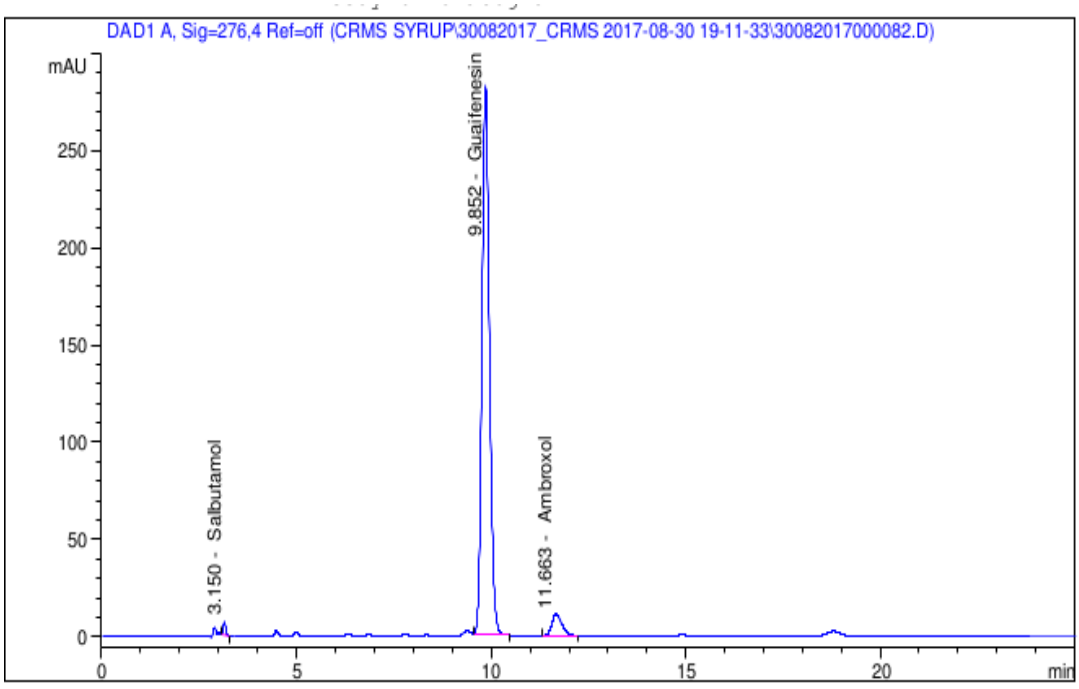
Sample_24Hrs



Sample_36Hrs



Sample_48Hrs



8. DISCUSSION

8.1 Method development

Several mobile phase compositions were tried to get good optimum resolutions of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride peaks. The mobile phase containing Sodium dihydrogen phosphate buffer+TEA (pH3.0):Acetonitrile:Methanol (65:10:25 %v/v) was selected because it gave sharp peaks with good resolution, minimum tailing and satisfactory retention time. Both the drugs having appreciable absorbance at 276nm and therefore 276nm was selected as the detection wavelength.

The working standard solutions of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride were injected separately. The retention time of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride was found to be 3.157min, 9.949min and 11.883min respectively when injected as individual compounds.

8.2 Validation of the method

The system suitability parameters were studied from the chromatogram to ascertain the suitability of the proposed method. The number of theoretical plates was found to be 14133 for Salbutamol and 13119 for Guaifenesin and 9978 for Ambroxol indicating the suitability of the method. The tailing factor was found to be 1.1 for Salbutamol and 1.1 for Guaifenesin and 1.3 for Ambroxol indicating good symmetry. The obtained resolution between Salbutamol and Guaifenesin was 29.90 and between Guaifenesin and Ambroxol was 4.69 indicating good and complete separation of three drugs (presented in table 4).

The accuracy of the method was determined by recovery experiments. The recovery study was carried out at 50%, 100% and 150% level. The percentage recovery and mean percentage of the recovery were calculated. The results showed percentage recovery of 100.5% to 101.6% for Salbutamol and 98.4% to 98.8% for Guaifenesin and 100.1% to 100.8% for Ambroxol was in agreement to the acceptance criteria 98% to 102%. From the data obtained, the recoveries of standard drugs were found to be accurate (presented in table 5&6&7).

The precision of system and method was determined by replicate injections of standard drug solution. In the system precision the %RSD of peak area was found to be 1.0 for Salbutamol and 0.3 for Guaifenesin and 0.5 for Ambroxol. In the method precision the

%RSD of assay was found to be 0.3 for Salbutamol and 0.06 for Guaifenesin and 0.06 for Ambroxol. The values of %RSD for precision study obtained were well within the acceptance criteria less than 2%. Thus the method providing high degree of precision (presented in table 8&9).

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions. The %RSD of peak area was found to be 0.2 for Salbutamol and 0.09 for Guaifenesin and 0.07 for Ambroxol (presented in table 10). Thus the results were found to be highly reproducible in spite of variations in the conditions.

To evaluate the linearity of method, the standard drug solutions of varying concentrations ranging from 50 % to 150 % of the targeted level of the assay concentration were examined by the proposed method. The peak area and concentration were plotted to get a standard calibration curve. The correlation coefficient was found to be 0.9994 for Salbutamol and 0.9999 for Guaifenesin and 0.9998 for Ambroxol. The linearity was obtained in the concentration range of 5-15 µg/mL Salbutamol and 250-750 µg/mL for Guaifenesin and 75-125 µg/mL for Ambroxol (presented in table 11). The calibration graph shows that linear response was obtained over the range of concentrations used in the assay procedure. The obtained data demonstrates that the methods have adequate sensitivity to the concentrations of the analytes. The range demonstrates that the method is linear outside the limits of expected use.

The robustness of the method was studied by carrying out experiments by changing experimental conditions flow rate and wavelength ratio. No significant effect on chromatographic resolution was seen and hence the developed method is said to be robust (shown on table 12-19).

The specificity of the method was studied by the values obtained while sample stored under relevant stress conditions. The procedure was unaffected by the presence of impurities (shown on table20&21).

The solution stability of the method, the standard and sample solutions were prepared vand injected for several days and check the similarity factor for standard solution and absolute % difference for sample solution. The similarity factor for standard solution were found to be, Initially 1.01, after 24 Hrs 1.00, after 36 Hrs 0.99 and after 48 Hrs 1.01

for Salbutamol and Initially 1.00, after 24 Hrs 1.01, after 36 Hrs 1.01 and after 48 Hrs 1.00 for Guaifenesin and Initially 1.00, after 24 Hrs 1.01, after 36 Hrs 1.01 and after 48 Hrs 1.00 for Ambroxol. The values for solution stability of standard was within the limit of 0.98-1.02. Thus, the solution stability of standard passes (shown on table 22). The absolute % difference for sample solution were found to be, Initially NA, after 24 Hrs 0.4, after 36 Hrs 0.2 and after 48 Hrs 0.1 for Salbutamol and Initially NA, after 24 Hrs 0.4, after 36 Hrs 0.6 and after 48 Hrs 0.3 for Guaifenesin and Initially NA, after 24 Hrs 0.3, after 36 Hrs 0.5 and after 48 Hrs 0.2 for Ambroxol. The values for solution stabily of sample was within the limit of . Thus, the solution stability of sample passes (shown on table 23).

Table 24 Validation data

Parameters		**Salbutamol Sulphate**	**Guaifenesin**	**Ambroxol hydrochloride**
Accuracy		% Recovery = 101.2	% Recovery = 98.6	% Recovery = 100.5
Linearity		R^2 = 0.9994	R^2 = 0.9999	R^2 = 0.9998
Range		05-15 µg/mL	250-750 µg/mL	75-225 µg/mL
System Precision		%RSD = 1.0	%RSD = 0.3	%RSD = 0.5
Method precision		%RSD = 0.3	%RSD = 0.06	%RSD = 0.06
Intermediate precision		%RSD=0.21	%RSD=0.09	%RSD=0.07
Robustness (Effect of flow)		%RSD=0.71	%RSD=0.07	%RSD=0.24
Robustness (Effect of wavelength)		%RSD=0.57	%RSD=0.07	%RSD=0.08
Solution stability	Standard	Similarity factor=1.01	Similarity factor=1.01	Similarity factor=1.01
	Sample	% difference=0.2	% difference=0.4	% difference=0.4

8.3 Application of the method for assay of sample

The method was applied for the assay of sample i.e. marketed liquid dosage form of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride. The average amount of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride present in marketed oral liquid dosage form was found to be 1.01mg, 49.68mg and 14.98mg respectively. Label claim for Salbutamol 1mg, Guaifenesin 50mg and that of Ambroxol hydrochloride 15mg.

9. SUMMARY AND CONCLUSION

A RP-HPLC method was developed with mobile phase system Sodium dihydrogen phosphate buffer pH 3.0: Acetonitrile: Methanol in the ratio of 65:10:25 with the flow rate of 1 mL/min. Detection was carried out at 276 nm. Quantitation was done by external standard method with the above mentioned optimized chromatographic condition. This system produced sharp peaks with good resolution, minimum tailing and satisfactory retention times of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride were found to be 3.157, 9.949 and 11.883 minutes respectively indicating the suitability of system.

The developed method was validated for various parameters accuracy, precision, linearity, robustness, specificity and solution stability as per ICH guidelines.

The accuracy of the method was in agreement to the acceptance criteria. The results indicate satisfactory accuracy of the method.

Precision of the developed method was studied under system precision, method precision and intermediate precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise.

The linearity was obtained in the concentration range of 5-15 µg/mL Salbutamol sulphate, 250-750 µg/mL for Guaifenesin and 75-225 µg/mL. The correlation coefficient was found to be 0.9994 for Salbutamol, 0.9999 for Guaifenesin and 0.9998 for Ambroxol hydrochloride which indicates excellent correlation between response factor Vs concentration of standard solutions.

The robustness of the method was studied. The results indicate that the method was robust and did not show significant effect on chromatographic resolution.

The specificity of the method was studied by the values obtained while sample stored under relevant stress conditions. The procedure was unaffected by the presence of impurities.

The method was applied for the assay of sample i.e. marketed tablet dosage form of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride. The assay results conformed to the label claim of the dosage form.

The developed method has the following advantages when compared to reported methods:

- No tedious extraction procedure required.
- Suitable for analysis of raw materials and formulations.
- Indicates stability of the drugs.

Hence the developed method for simultaneous estimation of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride said to be rapid, simple, accurate, precise, sensitive, robust and specific that can be successfully applied for the routine analysis of Salbutamol sulphate, Guaifenesin and Ambroxol hydrochloride in their marketed oral liquid dosage form.

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